An investigation into genome-scale ordered RNA structure (GORS) in murine norovirus and other positive-stranded RNA viruses

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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Abstract

Genome-scale ordered RNA structure (GORS) was first identified in 2004. It refers to the presence of secondary structure throughout the length of the RNA genomes of certain genera of RNA virus families, as predicted by bioinformatic analysis. It was also observed that the viruses containing GORS were able to establish persistent infections in their natural hosts, raising the possibility that the presence of GORS could play a role in viral avoidance of the innate immune system.

This thesis describes the first study of GORS and its possible role in persistence. Two GORS viruses have been studied, equine rhinitis A virus (ERAV) and murine norovirus (MNV). A 55% seroprevalence of ERAV has been determined in a cohort of Scottish horses indicating a wide exposure to the virus. Equine faecal samples were screened for ERAV by PCR with the intention of identifying a virus, possibly from a persistently infected animal, which would not have undergone any cell culture adaptations as laboratory strains have. Newly identified viruses would then be sequenced, their secondary structures predicted and further studies carried out. Unfortunately, none of the 50 faecal samples screened were positive and clinical isolates of ERAV provided by the Animal Health Trust were sequenced but were identical to laboratory strains, so the study then focussed on MNV. Prevalence of MNV in laboratory mice was determined by PCR of faecal samples to be 67%. MNV was also discovered in the faeces of a pet shop mouse and a wild wood mouse (*Apodemus sylvaticus*). The complete genomes of 4 laboratory mouse MNVs, the pet shop mouse and wood mouse MNVs were sequenced. Phylogenetic analysis showed the wood mouse MNV had a p distance of 23% from other MNVs, although the laboratory mice and pet shop mouse were closely related to other MNVs. Structural analysis of the genomes of 6 sequenced MNVs, including the wood mouse virus, showed all were GORS viruses. A laboratory strain of MNV, MNV-3, was serially passaged in RAW 264.7 cells to test the hypothesis that in an animal with an intact immune system, there is a pressure for GORS viruses to maintain their genomic RNA structure as a means of immune avoidance, and that cell culture adaptation would attenuate the degree of secondary structure. The complete genome of passage 33 was sequenced,

which revealed 7 base mutations, a mutation rate of 0.1 %, which was not considered significant enough to have affected the degree of secondary structure.

In order to assess if structured and unstructured RNA behaved differently in cells, replication deficient RNA transcripts were made from the infectious clones of a panel of GORS and non-GORS viruses. These transcripts were electroporated into cells and their rate of decay measured, but there was no difference between the GORS and non-GORS transcripts. The full length and 4 kilobase transcripts were transfected into NIH3T3 cells and the degree of interferon-β induction measured by quantitative PCR and a luciferase reporter assay. The IFN-β response differed across the panel of viruses, and although none of the GORS viruses induced strongly, the non-GORS viruses were variable in their ability to induce an IFN-β response, some inducing strongly, other not at all. This result indicates that during exposure of viral genomes in the cytoplasm during infection, GORS-virus RNAs are unlikely to induce an interferon response, possibly contributing to their ability to persist. It is unclear why some non-GORS-viruses failed to induce IFN and there are likely to be other contributory factors.

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Abbreviations

2’5’OAS	2’5’ oligoadenylate synthetase
A	Adenosine
ADAR	Adenosine deaminase
BV/BUNV	Bunyamwera virus
C	Cytosine
CaCV	Canine calicivirus
CARD	Caspase recruitment domain
Cardif	CARD adaptor IFN-β
cDNA	complementary DNA
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CRE	*cis*-acting replication element
CTD	C terminal domain
CTL	Cytotoxic T-Lymphocyte
D-MEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
dNPT	Deoxy-Nucleoside Triphosphate
dsDNA	Double Stranded DNA
dsRNA	Double-stranded RNA
E11	Echovirus 11
EDTA	Ethylenediaminetetraacetic acid
EEEV	Eastern Equine Encephalitis Virus
eGFP	Enhanced Green Fluorescent Protein
EMCV	Encephalomyocarditis Virus
ER	Endoplasmic Reticulum
ERAV	Equine rhinitis A virus
FCS	Foetal Calf Serum
FCV	Feline calicivirus
FMDV	Foot-and-Mouth Disease Virus
G	Guanosine
GBV-C	GB virus-C
GORS	Genome-scale ordered RNA structure
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
I	Inosine

IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IPS-1	IFN promoter stimulator
IRAK	Interleukin-1 receptor-associated kinase
IRES	Internal Ribosome Entry Site
IRF	IFN regulatory factor
IRF-3	IFN regulatory factor 3
ISG	IFN stimulated gene
JAK	Janus kinase
Kb	Kilobase
LCMV	Lymphocytic Choriomeningitis Virus
M.O.I	Multiplicity of Infection
MAVS	Mitochondrial activated signalling
MCP	Monocyte chemotactic protein
MDA-5	Melanoma differentiation associated gene 5
MDCK	Madin-Darby canine kidney
MFE	Minimum free energy
MFED	Minimum free energy difference
MHC	Major Histocompatibility Complex
MHV	Mouse Hepatitis Virus
MLN	Mesenteric lymph node
MNV	Murine norovirus
MV	Measles Virus
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor κB
NK cell	Natural killer cell
nsP	Non Structural Protein
OD	Optical Density
ORF	Open Reading Frame
P	Protruding domain
PAMP	Pathogen associated molecular pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid dendritic cells
PEMV	Pea enation mosaic virus
PKR	dsRNA-activated protein kinase
PML NB	Promyelocytic leukaemia nuclear bodies
PRR	Pattern recognition receptor
PV	Poliovirus

RAG	Recombination activating gene
RD	Rhabdomyosarcoma
RHDV	Rabbit haemorrhagic disease virus
RIG-I	Retinoic acid inducible gene-I
RIP	Recptor interacting protein
RNA	Ribonucleic Acid
RT	Reverse transcription
RV	Rubella virus
S	Shell domain
SFV	Semliki Forest Virus
SINTBAD	Similar to NAP1 TBK1 adaptor
STAT	Signal Transducers and Activators of Transduction
SV	Sindbis Virus
T	Thymine
TAB	TAK binding proteins
TAK	TGF-β activated kinase
TBE	Tris borate EDTA
TBK	TANK-binding kinase
$TCID_{50}$	Tissue culture infective dose 50
TGF-β	Transforming growth factor β
TLR	Toll-Like Receptor
TMEV	Theiler's Murine Encephalitis Virus
TNF	Tumour Necrosis Factor
TRAF	TNF receptor associated factor
TRIF	Toll IL-1 domain-containing adaptor inducing IFN
TRIM	Tripartite motif containing
Tyk	Tyrosine kinase
U	Uracil
UTR	Untranslated region
VEEV	Venezuelan Equine Encephalitis Virus
Visa	Virus-induce signalling adaptor
VLPs	Virus-Like Particles
VPg	Viral protein genome-linked
VSV	Vesicular stomatitis virus
VZV	Varicella Zoster Virus
WEEV	Western Equine Encephalitis Virus
WT	Wild-type

Chapter 1 Introduction

Chapter 1 General introduction

Genome-scale ordered RNA structure (GORS) is a phenomenon first described by Simmonds *et al*. in 2004. Using bioinformatic analysis of viral RNA genomes, based on energy minimising algorithms, regions of secondary structure were predicted. The term "GORS" describes the occurrence of extensive RNA formation throughout the length of the RNA genomes in many families and genera of positive-sense RNA virus families. While the high degree of structure in these genomes appeared to be unrelated to fundamental function in viral replication, there was an observed association between the presence of GORS and the ability of the virus to establish persistent infections. This thesis presents the first investigation of the biological effect of GORS by studying aspects of the biology of predicted GORS viruses and the interactions between structured and unstructured viral RNA with cells.

1.1 *Caliciviridae*

The *Caliciviridae* are a family of non-enveloped, single-stranded, positive-sense RNA viruses. Members of this family cause a range of human and veterinary diseases. There are four genera within the family, each named after a typical member of the genus (Green *et al.*, 2000); the four genera are:

i) *Vesivirus* e.g. *Vesicular exanthema of swine virus, Feline calicivirus*
ii) *Lagovirus* e.g. *European brown hare syndrome virus, Rabbit haemorrhagic disease virus*
iii) *Norovirus* e.g. *Norwalk virus, Murine norovirus*
iv) *Sapovirus* e.g. *Sapporo virus*

Recent studies have proposed two new genera; bovine enteric virus has been assigned to a genus with the proposed names of *Becovirus* or *Nabovirus* (Oliver *et al*., 2006); and the Tulane virus, isolate from rhesus macaques, to a genus named *Recovirus*, derived from rhesus enteric calicivirus (Farkas *et al*., 2008).

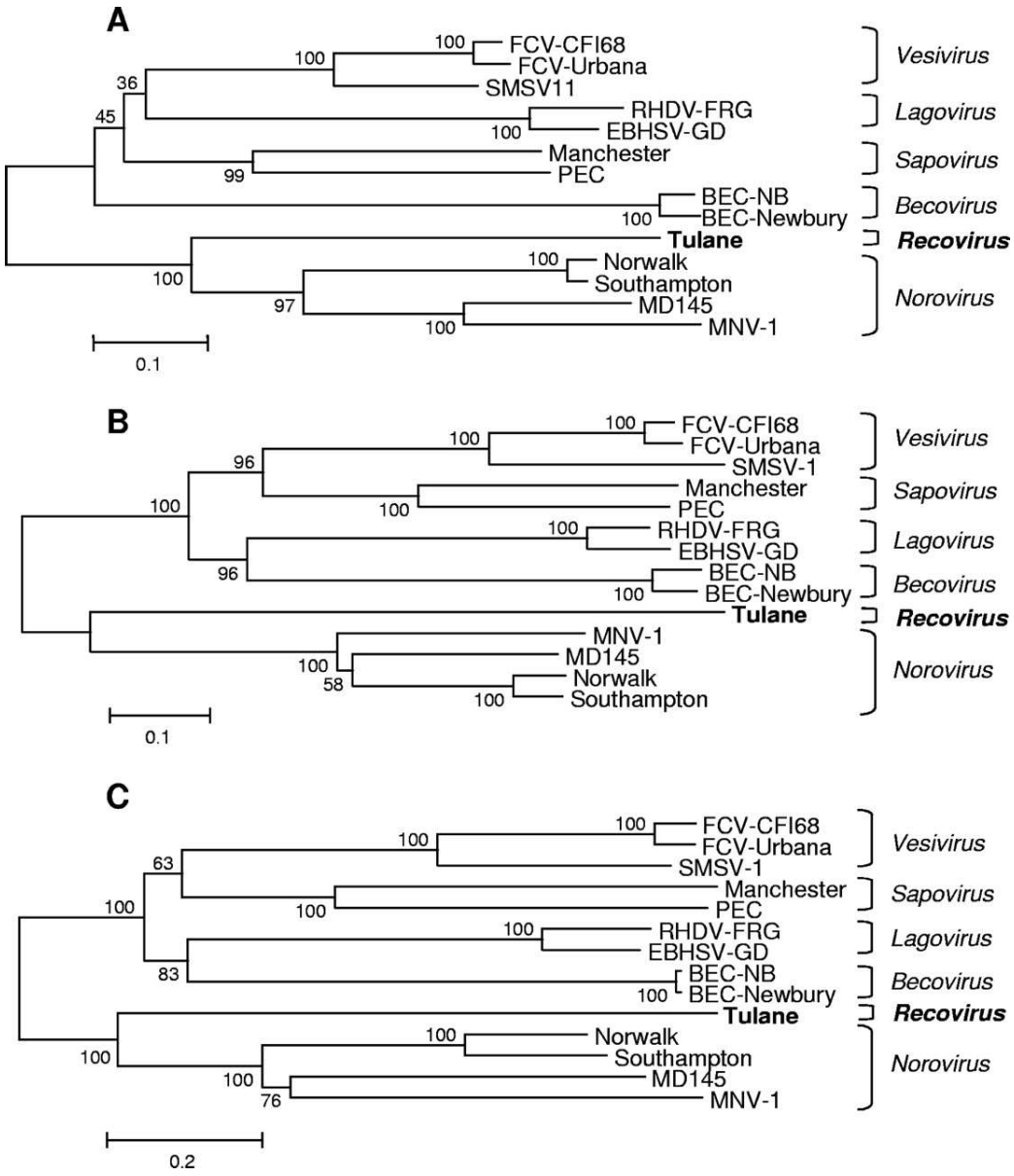


Figure 1.1 Phylogenetic trees showing calicivirus genera based on sequences alignments of: A – NTPase; B – polymerase; C- VP1. The most recently designated genera, *Becovirus* and *Recovirus* included. Reproduced from Farkas *et al*., 2008.

The calicivirus virion is a small (27 - 40 nm) particle. The name calicivirus is derived from the appearance of the 32 cup-shaped depressions on the surface, the Latin word "calyx" providing the root. The Caliciviridae family contains a number of important veterinary and medical pathogens. Feline calicivirus (FCV) is an important pathogen of cats, causing an upper respiratory tract infection with stomatitis. Rabbit haemorrhagic disease virus (RHDV), as the name suggests, causes a haemorrhagic disease in rabbits. Human noroviruses cause acute gastroenteritis and are discussed in more detail below. The genera

of *Caliciviridae* share similar virion structures, genome arrangements and replication strategies (Green, 2007; Fields Virology 5th Edition, Ed by Knipe and Howley).

1.2 Noroviruses

1.2.1 History of noroviruses

The genus *Norovirus* took its name from the first discovered member of the genus, Norwalk virus, which cause acute gastroenteritis in humans. Before the discovery of the virus, episodes of acute non-bacterial gastroenteritis were known as "winter vomiting disease", first described by Zahorsky in 1929. Throughout the 1940s and 1950s volunteer studies in the United States and Japan demonstrated that the disease was transmissible after administration of bacteria-free faecal suspensions (Reimann *et al*., 1945; Jordan *et al.*, 1953; Fukumi *et al.*, 1957) indicating a viral aetiology. An outbreak of gastroenteritis in Norwalk, Ohio in 1968 at school affected 50 % of the pupils and teachers (116 / 232). The disease had an incubation period of ~48 h and the clinical course lasted ~24 h. The clinical signs of the disease were acute nausea, vomiting, diarrhoea and abdominal cramps. This was described as an outbreak of winter vomiting disease due to its similarity to the disease described in 1929 (Adler and Zickl, 1969). The aetiological agent was identified by the discovery of a 27 nm particle, by immune electron microscopy, in the faecal filtrate of one of the case from the Norwalk school outbreak (Kapikian *et al.*, 1972). This was the discovery of Norwalk virus, which became the prototype member of the genus now known as *Norovirus*.

1.2.2 Norovirus classification

The classification of noroviruses has been determined predominantly by genetic analysis as, until the discovery of a cell culture system for murine norovirus (MNV), propagation of noroviruses in cell culture had not been possible. Phylogenetic analysis of the sequence of norovirus VP1 sequence divided the genus into five genogroups: genogroups I, II and IV contain human noroviruses, genogroup III contains bovine noroviruses and genogroup V contains MNV. Genogroup II also contains porcine noroviruses (Figure 1.1). The genogroups are also divided into genotypes, also known as phylogenetic clusters (Green *et al.*, 1999; Ando *et al.*, 2000; Vinje *et al.*, 2000; Zheng *et al.*, 2006). Human noroviruses diverge by up to 45 % at the nucleotide level (Zheng *et al.*, 2006)

and MNVs diverge by up to 13 % at the nucleotide level that comprise a single genotype and genogroup (Thackray *et al.*, 2007). The diversity of noroviruses may in part be due to the fact that the RNA-dependent RNA polymerases of RNA viruses have no proofreading function. It has also been shown that recombination events at the overlap between ORF1 and ORF2 of MNV also lead to the genetic diversity of noroviruses (Worobey and Holmes, 1999; Ambert-Balay *et al.*, 2005; Müller *et al.*, 2007).

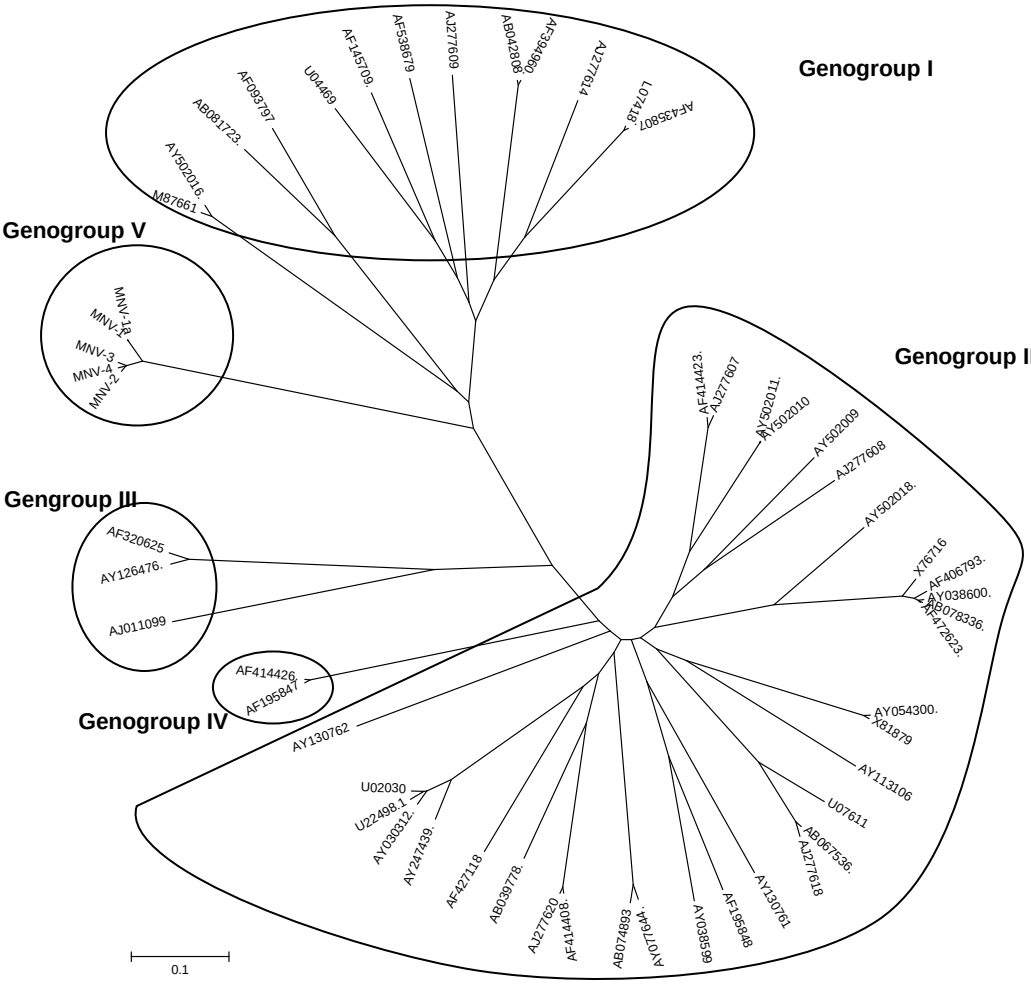


Figure 1.2: Phylogenetic tree of *norovirus* genus showing classification into 5 genogroups based on complete capsid protein (VP1) sequences. Genogroup I contains human viruses; genogroup II, human and porcine; genogroup III, bovine; genogroup IV human and canine; genogroup V, murine

1.2.3 Norovirus virion structure

The norovirus virion has a diameter of ~38 nm, and exhibits T=3 icosahedral symmetry made up of 90 dimers of the capsid protein. The capsid structure contains arch-like capsomeres, creating large hollows at the icosahedral 5- and 3-fold axes (Prasad *et al.*, 1994; Prasad *et al.*, 1999). The capsid protein (VP1) self-assembles into virus-like particles which resemble native capsids in both appearance and size (Jiang *et al.*, 1992). The structure of this protein is modular, consisting of the lower shell domain (S) and protruding domain (P) connected by a flexible hinge. The protruding domain is then split further into the central stem domain (P1) and distal globular domain (P2). It is the protruding domains that make up the arch-like structures, with the S domain acting as an icosahedral scaffold (Chen *et al.*, 2006). Katpally *et al* showed that the protruding domain is lifted off the shell by ~16Å and rotated in a clockwise direction by 40°, forming new interactions at the base of P1, creating a cage-like structure that engulf the shell domains (Katpally *et al.*, 2008). The amino acid sequence of the S domain is relatively conserved throughout the noroviruses, whereas the P2 domain is the most variable, indicating its role in receptor binding and antigenicity (Prasad *et al.*, 1999; Nilsson *et al.*, 2003; Tan *et al.*, 2003).

1.2.4 Norovirus genome structure

Norovirus has an RNA genome of ~7.5 kilobases, the genome is a positive-sense, single-stranded RNA molecule. The genome contains 3 well-defined open reading frames (ORF), and a fourth of uncertain function has recently been postulated in MNV, but not in human, bovine or porcine noroviruses (Thackray *et al*.2007),. The 5' end of the genome is covalently bound to a viral protein called the VPg protein. ORF1 encodes a large polyprotein which is subsequently cleaved by viral proteases into the non-structural (NS) proteins (Liu *et al.*, 1996; Blakeney *et al.*, 2003; Sosnovtsev *et al.*, 2006). ORF2 encodes the major capsid protein, also known as the viral protein VP1 which makes up the S and P domains (Jiang *et al.*, 1990; Prasad *et al.*, 1999). ORF3 encodes the small basic protein, VP2, which appears to have a role in the stability of the virion (Bertolotti-Ciarlet *et al.*, 2003; Glass *et al.*, 2000). ORF 2 and 3 are thought to be encoded by a sub-genomic RNA. There is also a 3' untranslated

region in noroviruses, consisting of a stem loop of 47 nucleotides and a poly(A) tail of 24 nucleotides (Gutierrez-Escolano *et al.*, 2003).

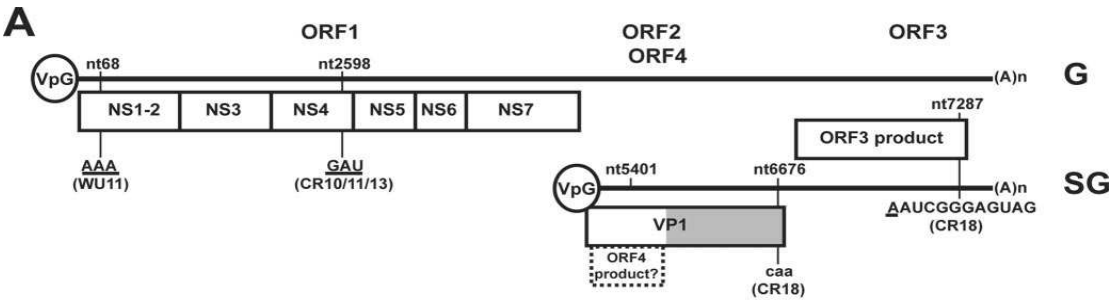


Figure 1.3: Genome of MNV showing ORF1, the sub-genomic ORF2 and ORF3 with putative ORF4 and proteins encoded. Taken from Thackray *et al.*, 2007.

1.2.5 Norovirus attachment and cell entry

Noroviruses are usually transmitted orally, and the virions are acid-stable which enables them to pass through the stomach and cause enteritis in humans. Human noroviruses have been shown to bind histo-blood group antigens and to carbohydrate antigens in the gut, specifically Lewis type antigens (Hutson *et al.*, 2003). The part of the capsid that interacts with the cellular receptor has also been shown to be the hyper variable P2 domain (Lochridge *et al.*, 2005). Recently it has been reported that the receptor for MNV has been identified as ganglioside-linked terminal sialic acid moieties on murine macrophages (Taube *et al.*, 2009). After attachment of the virus and entry into cells the genome must be uncoated. Bhella *et al* showed that the capsid of feline calicivirus (FCV) (a member of the genus *Vesivirus* of the family *Caliciviridae*) underwent a conformational change induced by binding to its cellular receptor (Bhella *et al.*, 2008). FCV, however, has also been shown to enter cells by clathrin-mediated mechanism in acidified endosomes (Stuart and Brown, 2006), whereas MNV entry into permissive macrophages and dendritic cells is pH independent (Perry *et al.*, 2009).

1.2.6 Calicivirus / Norovirus replication

As noroviruses have proven difficult to grow in cell culture until the recent success in growing MNV in RAW264.7 cells, much of what is known about calicivirus replication has come from work on FCV. This section will discuss calicivirus replication based on work on FCV and MNV. As single-stranded, positive-sense RNA viruses, calicivirus RNA acts as a messenger RNA when released into cells. The RNA is translated by using the cellular machinery for the creation of progeny virus particles which are then released from the cell. Unlike other families of RNA viruses, viruses of the family *Caliciviridae* have neither a long 5' UTR, with highly structured regions such as internal ribosome entry sites (IRES), nor a 5' 7-methylguanosine cap with which to interact with host ribosomes. The VPg protein at the 5' end of the genome has been shown to bind to translation initiation factors in infection by Norwalk virus (Daughenbaugh *et al.*, 2003) FCV (Goodfellow *et al.*, 2005; Chaudhry *et al.*, 2006) and MNV (Daughenbaugh *et al.*, 2006). MNV1 VPg, for example, was co-precipitated with eIF4GI and eIF4E from infected murine macrophages (Daughenbaugh *et al.*, 2006). Translation of the genome produces a large polyprotein. This protein then undergoes proteolytic cleavage by a virus-encoded protein called 3C-like proteinase (3CL Pro), this process produces the non-structural proteins. In the MNV polyprotein there are five cleavage sites in the ORF1 protein: ^{341}E/G^{342}, ^{705}Q/N^{706}, ^{870}E/G^{871}, ^{994}E/A^{995} and ^{117}Q/G^{1178}. These cleavages produce six mature non-structural proteins: NS1-2 (Nterm), NS3 (NTPase), NS4 (3A-like), NS5 (VPg), NS6 (Pro) and NS7 (Pol) (Sosnovtsev *et al.*, 2006).

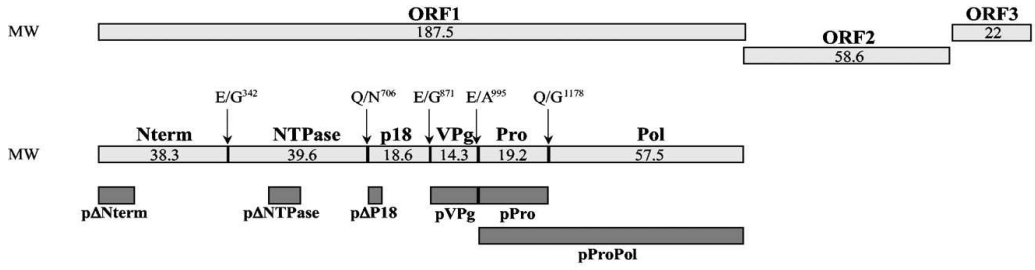


Figure 1.4: Diagram of MNV polyprotein including cleavage sites and non-structural proteins of ORF1. Taken from Sosnovtsev *et al.*, 2006.

RNA replication then occurs by the action of the RNA-dependent RNA polymerase. This takes place from the polyadenylated tail of the 3' end of the genome which creates a negative-sense strand of RNA, the reverse complement of the genomic RNA. In cells infected with FCV it has been shown that there are two double-stranded RNA molecules corresponding to the genomic (8.2 kb) and sub-genomic (2.4 kb) RNAs. The negative strands then act as a template for the production of progeny genomic RNAs (Neill and Mengeling, 1988). FCV viral RNA replication is associated with host cell membranes (Green *et al.*, 2002). The positive-strand subgenomic RNA then acts as a template for the translation of ORF2 and ORF3, producing the major capsid protein VP1, and the small basic protein VP2. As noted above, the capsid proteins undergo self-assembly very readily to produce virus-like particles, giving an indication to how the virus particles assemble. The packaging signal for norovirus RNA may be found in the non-structural region of the genome, or that the non-structural proteins, or even VPg, may be necessary for packaging of RNA (Asanka *et al.*, 2005). Recent work has revealed that MNV-1 replication is associated with cellular membranes similar to many other RNA viruses; MNV-1 replication complexes have been identified in a perinuclear location involving membranes derived from the endoplasmic reticulum, Golgi and endosome (Hyde *et al.*, 2009). It has been suggested that caliciviruses induce cells to undergo apoptosis as a means of releasing progeny virus. Another feature of MNV-1 infection of RAW264.7 cells, is that it is able to inhibit the expression of the antiapoptotic protein survivin, and that there was an increase in levels of caspase-3, a factor involved in the execution of cellular disassembly. Changes were also noted in the nuclear chromatin and DNA fragmentation, other markers of apoptosis (Bok *et al.*, 2009). These observations support the theory that noroviruses are able to use apoptosis as a mechanism for virus release.

1.2.7 Norovirus infections of humans

Norovirus infections in humans affect people of all ages (Rockx *et al.*, 2002) and can occur all year round, but there is a cold weather peak in incidence (Mounts *et al.*, 2000). The illness in humans is generally a mild, acute onset gastroenteritis characterised by nausea, vomiting, diarrhoea and abdominal cramps. Vomiting is the predominant clinical sign in children over 1 year old,

and diarrhoea is the main feature in children under 1 year and adults (Kaplan *et al.*, 1982). The cause of the clinical disease typically lasts 2-3 days, although this may be longer during hospital outbreaks (Lopman *et al.*, 2004). Transmission is by the faecal-oral route, by aerosolisation of viral particles from infectious vomit or person-to-person contact. These routes of transmission enable the rapid spread of norovirus during outbreaks in enclosed environments such as hospitals, schools and cruise ships (Widdowson *et al.*, 2005; Becker *et al.*, 2000; Gunn *et al.*, 1980). Outbreaks usually begin after exposure to contaminated food or water (Kaplan *et al.*, 1982b). Spread of the virus is made easier by the fact that it has a very low infectious dose, and the probability of infection of a single Norwalk virus particle estimated to be close to 0.5 (Teunis *et al.*, 2008), the prolonged viral shedding after resolution of clinical signs and the stability of the viral particle in high concentrations of chlorine (Duizer *et al.*, 2004). Immunity to norovirus has been demonstrated in volunteer studies, but it is short-lived (6-12 weeks), and subjects were susceptible to infections when challenged 2-3 years later (Parrino, *et al.*, 1977). Susceptibility to norovirus infection is determined by the presence of specific human histo-blood group antigen (HBGA) receptors. These are present in the gut which is the presumed site of virus replication (Lindesmith *et al.*, 2003; Hutson *et al.*, 2004).

1.2.8 Noroviruses in animals

Since their recognition as significant human pathogens, noroviruses have also been detected in other species, such as cattle, pigs and mice. Norwalk-like virus genes were discovered in the caecal contents of healthy pigs in Japan in 1998. There was 58.2 % to 59.9 % similarity between Norwalk virus and those detected in the pigs, and the new viruses were placed in the norovirus genogroup II (Sugieda *et al.*, 1998). Small, round viruses were first identified in the UK by electron microscopy in calf diarrhoea in 1978 (Woode and Bridger, 1978) and in Germany in 1980 (Gunther and Otto, 1987). The German virus, known as Jena virus, was sequenced in 1999 and was shown to have a markedly similar genome organisation to Norwalk virus, and phylogenetic studies of the RNA polymerase sequence placed it with other noroviruses (Liu *et al.*, 1999; van Der Poel *et al.*, 2000). Bovine noroviruses were later classified as genogroup III (Liu *et al.*, 1999; Oliver *et al.*, 2003). Porcine noroviruses appear to cause only

sub-clinical infections (Wang *et al.*, 2007) but are relatively prevalent, 17 % - 20 % (Reuter *et al.*, 2007; Wang *et al.*, 2006). Porcine noroviruses are often monitored due to their genetic relatedness to human noroviruses and potential for zoonotic spread (L'Homme *et al.*, 2009). Bovine noroviruses are associated with diarrhoea in gnotobiotic calves (Woode and Bridger, 1978) although their impact in the wider cattle population is not clear. A recent study has shown the prevalence in Belgium to be 7.5 % by faecal RT-PCR (Mauroy *et al.*, 2009).

1.2.9 Murine norovirus

Murine norovirus was first described by Karst *et al.* in 2003. It was observed that laboratory mice deficient in the recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT1) (RAG/STAT$^{-/-}$ mice) would sporadically succumb to an unknown pathogen. RAG2 plays an essential role in the development of T and B lymphoid cells and their diversity of cell surface receptors, enabling them to recognise numerous pathogens; thus it plays a crucial role in the development of adaptive immunity (reviewed by Sobacchi *et al.*, 2006). These mice were severely immunocompromised and the infection would produce meningoencephalitis, cerebral vasculitis and pneumonia. The pathogen was more virulent in mice which were deficient in the interferon (IFN) αβ and interferon γ receptors. The aetiological agent was suspected to be viral as it could be passed through a 0.2 µm filter. Rational difference analysis was carried out and the sequences obtained were similar to calicivirus genomes (Karst *et al.*, 2003). The ability of this virus to cause disease was then tested in mice which had normal expression of STAT1 but were deficient in RAG1 or RAG2. The mice were inoculated by intracranial, intranasal and peroral routes, but 90 days later a significant number of mice had not succumbed to the infection. The RAG$^{-/-}$ mice in fact became persistently infected, indicating that T and B cell adaptive immunity was not necessary for protection against the lethal disease. It was hypothesised that the innate immune system was primarily responsible for protection against the virus. Mice with various parts of the innate immune system, IFNαβ and IFNγ receptors, protein kinase RNA-activated (PKR) or inducible nitric oxide synthetase (iNOS), knocked out were challenged again with the virus. Mice with IFNαβ and IFNγ receptors knocked out were 10,000 fold more susceptible, showing that

interferons were essential for resistance (Karst *et al.*, 2003). Macroscopic pathological changes present in the affected RAG/STAT$^{-/-}$ mice included splenomegaly and multifocal pale foci on the liver. Hepatitis ranging in severity from mild to severe has been described consisting of focal to diffuse inflammatory infiltrates of mononuclear cells and neutrophils. Focal interstitial pneumonia, consisting of macrophages within the alveolar air-spaces and walls, was seen in some mice but was usually mild. Immunohistochemistry against an MNV antigen identified infected cells to be those of macrophage or dendritic cell type in the liver (Kupffer cells), red pulp of spleen, intestinal lamina propria and mesenteric lymph nodes (Ward *et al.*, 2006). Infection in immunocompetent mice occurs, with MNV-1, which has been shown to replicate and disseminate to various parts of the body. Histological examination of normal mice infected with MNV1 has revealed: mild enteritis consisting of increased numbers of granulocytes; the spleens showed red pulp hypertrophy and white pulp activation at 72 hours post infection. MNV-1 was shown to be present in the spleen, lung, liver and lymph nodes. The function of STAT1 is to inhibit viral replication and thus the prevention of virus-induced apoptosis resulting in little spread of the virus, resulting in the absence of clinical disease in immunocompetent mice (Mumphrey *et al.*, 2007; Perdue *et al.*, 2007).

After the discovery of MNV-1, further MNVs were discovered and described. In 2006, Hsu *et al.* described the identification of MNV-2, MNV-3 and MNV-4. Immunocompetent mice from geographically separate laboratories in the United States were euthanased and their mesenteric lymph nodes (MLN) screened for MNV by a multiplex fluorescent assay. MLN material was inoculated onto permissive cells and 3 novel strains of MNV were isolated. It was also shown that these novel strains persisted in mice as MNV was detected in faeces by RT-PCR for up to 8 weeks post-infection, whereas MNV-1 only produced a transient infection which was quickly cleared (Hsu *et al.*, 2006). Further characterisation of the novel MNVs, from comparison of full length nucleotide sequences, showed that despite their different biological behaviours they had a high degree of sequence homology with MNV-1, but not with human or bovine noroviruses (Hsu *et al.*, 2007). Thackray *et al.* in 2007 conducted a study into MNV diversity by sequencing the complete genome of 21 novel MNVs and

compared them to the 5 sequences published at that time. They discovered 15 distinct strains of MNV, with up to 13 % divergence at the nucleotide level. These 15 strains, however, made up a single genogroup, genotype and serotype; there was also evidence of recombination within ORF2 in several of the MNV genomes (Thackray *et al.*, 2007). The study of MNV, and of noroviruses in general, was made easier with the development of a cell culture system which could support the growth of MNV. Efforts had been made to cultivate human noroviruses in the laboratory but were unsuccessful (Duizer *et al.*, 2004). The observation that MNV had been identified in the macrophage/dendritic cells in the liver, spleen and small intestine lead to the attempt to grow MNV in this cell type. Bone marrow derived macrophages and bone marrow derived dendritic cells were inoculated with MNV-1 stock, derived from the brains of the mice that had originally lead to the discovery of MNV (Karst *et al*., 2003). MNV-1 had a marked tropism for both cell types as infection produced cytopathic effect in both. The cell line RAW 264.7, a macrophage cell line transformed by Abelson leukaemia virus (Raschke *et al.*, 1978), was found to support MNV replication with visible CPE and plaque formation (Wobus *et al.*, 2004). This cell line has since become the standard means of growing MNV in laboratories. A further advance in the study of noroviruses has been the development of reverse genetics system for MNV. In 2007, Ward *et al* described the recovery of infectious MNV after the baculovirus delivery of MNV cDNA into human hepatoma cells using an inducible DNA polymerase promoter (Ward *et al.*, 2007). Chaudhry *et al.* also demonstrated the recovery of genetically defined MNV using the fowlpox virus T7 RNA polymerase system (Chaudhry *et al.*, 2007). The ability to manipulate the MNV genome in future will surely reveal a great deal about norovirus replication and pathogenesis.

1.2.10 Immunity to noroviruses

The identification of MNV in RAG/STAT$^{-/-}$ mice and subsequent studies lead to the conclusion that the innate immune system was primarily responsible for protection against fulminate clinical disease associated with MNV infection (Karst *et al.*, 2003; Mumphrey *et al.*, 2007). A functional innate immune system does not protect the mice from infection though, as several strains of MNV have been shown to infect mice sub-clinically and to persist in immunocompetent

hosts (Hsu *et al.*, 2006). These observations are consistent with the clinical course of human norovirus infections i.e. acute onset and short duration. The rapid resolution of the disease suggests that an adaptive immune response would not have time to have been invoked to play a role in the course of the disease; however, adaptive immunity does have a role to play in clearing the norovirus infection. Since MNV-1 infections of $RAG^{-/-}$ mice, which have impaired adaptive immunity but unaffected innate immunity, did not succumb to clinical disease, but had higher levels of viral RNA in lung, liver, spleen, proximal intestine, brain, blood and faeces 90 days after peroral inoculation (Karst *et al.*, 2003). The conclusion was that the adaptive immune system is necessary for the clearance of MNV.

The innate immune system will be discussed in more detail later in this introduction. Briefly, it consists of the detection of pathogen-associated molecular patterns by cellular receptors which lead to the production of type I interferons (IFN-αβ). The pattern recognition receptors (PRR) that initiate the response to viruses include: toll-like receptor 3 (TLR3) and the Rig-I-like helicases, RIG-I and MDA-5 (Takeuchi and Akira, 2007). IFN-αβ then acts in a paracrine fashion, binding to its cellular receptors and act via the JAK-STAT pathway to upregulate the expression of numerous genes which collectively produce the antiviral state. The antiviral molecules include: protein kinase R (PKR), 2' 5'oligoadenylate synthetase (OAS) and ISG15 (reviewed by Randall and Goodbourn, 2008). The pathogenicity of MNV-1 in $STAT^{-/-}$ mice demonstrates how significant this system is in protecting mice from disease. As MNV has a tropism for cells of the macrophage/dendritic cell lineage, a study was conducted by McCartney *et al.* in 2008 in which bone marrow derived dendritic cells (BMDC) from wild-type, $TLR3^{-/-}$ and $MDA5^{-/-}$ mice were infected with MNV-1. They showed that MDA-5 recognises MNV-1 which stimulates the production of type I IFN, along with IL-6, MCP-1 and TNFα. The $MDA\text{-}5^{-/-}$ cells had a severe deficit in IFN production, although $MDA\text{-}5^{-/-}$ mice were still able to clear MNV-1 infection. It was concluded that MDA-5 is the predominant sensor in BMDCs, but that other sensors may play a role in other cell types which are important during infection of an animal. It was also suggested that the 5' VPg is essential for MDA-5 recognition of MNV-1

(McCartney *et al.*, 2008). Changotra *et al* have recently elucidated part of the mechanism by which the IFN response is able to abrogate MNV infection. They have shown that there is an IFN-mediated blockage to MNV-1 replication after virus entry and genome uncoating, but before the translation of the non-structural polyprotein. The most likely explanation was considered to be that the production or accumulation of the non-structural polyprotein is being prevented by an IFN-induced product. They have also shown that PKR is not a requirement for type I IFN-induced block to virion production. A possible mechanism for the IFN antiviral effect is that it affects the association of the VPg with the cellular translation machinery, leading to a failure to translate viral genomes (Changotra *et al.*, 2009).

Antibodies generated against noroviruses have been shown to recognise epitopes on the protruding domain (P2) of the VP1 protein which contain conserved amino acids sequences across human, murine, bovine and porcine noroviruses (Lochridge *et al.*, 2005). A single amino acid substitution in the P2 domain of MNV was actually sufficient for escape from antibody neutralisation (Lochridge *et al.*, 2007). A recent study in which mice were infected with a high dose of MNV-1, followed by a secondary challenge 6 weeks later, revealed that there was no protection from a homologous virus (Liu *et al.*, 2009). Although the antibody response did not prevent disease in the rechallenged mice, a memory immune response was elicited as the virus was cleared more quickly on the second infection. The reasons for the lack of protection are not clear, although it could be due to inappropriate tolerance of the virus or a direct result of the infection of antigen presenting cells inhibiting their function (Liu *et al.*, 2009).

Recent studies into the effectiveness of vaccines against MNV have revealed more information about the nature of the adaptive immune response. Chachu *et al.* have recently demonstrated that vaccination by oral administration of live MNV-1 virus produced a long lasting immunity, up to 24 weeks post treatment. It was demonstrated that for that for vaccination to be effective the presence of CD4 T cells, CD8 T cells and B cells were necessary, and that the MNV VP1 protein contains epitopes for each cell type. The molecule perforin, a product of CD8 cytotoxic T cells was found to be important in the clearance of the virus

(Chachu *et al.*, 2008). This study was conducted in MNV-1, which is not a persistent strain; it is unclear whether this vaccine is effective in persistent strains of MNV. Another recent study by LoBue *et al.*, has shown that norovirus-like particles with alphavirus adjuvant particles, when administered to mice, produced cross-reactive and receptor-blocking antibodies, and protection against infection with heterologous MNVs (LoBue *et al.*, 2009).

The study of the role of the adaptive immune system in human norovirus infections has provided a picture of the nature of the response. Due to the historical difficulty in growing noroviruses in cell culture, human norovirus studies have often relied on clinical material from outbreaks or volunteer studies. Acute norovirus gastroenteritis in humans is characterised by an infiltration of mononuclear cells into the jejunum, a transient circulating lymphopaenia and the presence of serum antibody (Dolin *et al.*, 1975; Dolin *et al.*, 1976). Prevalence of norovirus antibodies in humans has been shown to be high in many countries (reviewed by Lopman *et al.*, 2002), e.g. 2,382 of 3,250 English serum samples (72.3 %) were seropositive (Gray *et al.*, 1993). Rechallenge studies to determine if the antibody response was protective against future infection produced a picture whereby some people develop a short-term immunity to rechallenge with the same agent, whereas others are protected for up to 42 months later (Parrino *et al.*, 1977; Blacklow *et al.*, 1979).

The cytokine responses to norovirus infections have been described in human norovirus infections. A study of a natural outbreak of human norovirus demonstrated increased levels of IL-2 and IFN-γ in faecal samples, suggesting a Th1 immune response (Ko *et al.*, 2006). Similarly, a volunteer study with Snow Mountain virus, a human norovirus, also stimulated the production IL-2 and IFN-γ (Lindesmith *et al.*, 2005). These cytokine profiles are consistent with the observation that the cytotoxic T cell system is important in the clearance of MNV-1 (Chachu *et al.*, 2008). There is still much to learn about the exact nature of the adaptive immune response to noroviruses in the way in which humoral and cell mediated immunity interact, and how some strains of MNV are able to persist whereas MNV-1 and human noroviruses are not.

1.2.11 Diagnosis of noroviruses

Early in the history of norovirus diagnosis the most reliable method was to administer faecal filtrates to volunteers. Noroviruses were first identified by the use of electron microscopy and immune electron microscopy (Kapikian *et al.*, 1972). Other diagnostics test followed in the following years: radioimmunoassay (Greenberg and Kapikian, 1978), enzyme immunoassay (Herrmann *et al.*, 1985) and Western blot (Hayashie *et al.*, 1989), which detected either norovirus antigen or antibody. With the cloning and sequencing of the Norwalk virus genome (Xi *et al.*, 1990; Matsui *et al.*, 1991) molecular diagnostic techniques were described. Noroviruses were detected in human stool samples by PCR (Jian *et al.*, 1992; Moe *et al.*, 1994; Le Guyader *et al.*, 1996; Atmar *et al.*, 2001; Vinje *et al.*, 2003). Real-time PCR was developed to detect and quantitate Norwalk virus in stools (Richards *et al.*, 2004; Kageyama *et al.*, 2003; Pang *et al.*, 2004). The same diagnostic methods have been extensively used to detect the noroviruses of other species

1.3 Viruses and innate immunity

The innate immune system of vertebrates consists of barriers protecting the body from microbial attack. These barriers may be anatomical e.g. the epidermis of the skin, or the cilia of the respiratory epithelium or based on secretions e.g. mucosal immunity associated with various epithelial linings and gastric acid secretions. The response of the innate immune system against invading pathogens is on-specific and often leads to the activation of the complement system and acute inflammation. Cells of the innate immune system include dendritic cells and tissue macrophages; these cells produce many cytokines, including interferons (IFN), are able to present antigens to lymphocytes and in so doing link the innate immune system to the adaptive. In order to respond to pathogens, the innate immune system is able to recognise pathogens that have invaded the host. Pathogens are recognised by pathogen associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs) (Kindt *et al*., 2007). PAMPs are molecular patterns present in pathogens but are usually absent from the host. It is the recognition of dsRNA, the most important PAMP produced during replication of RNA viruses, which will be discussed in relation to the relationship between the presence of GORS in viral genomes and the ability of those viruses to persist. This discussion will focus on the IFN-β system as the detection of dsRNA leads to the production of IFN-β via PRRs such as toll-like receptor -3, RIG-I and MDA5 during viral replication, which leads to the establishment of the anti-viral state. It has been hypothesised that GORS leads to persistence by enabling the avoidance of recognition of viral RNA by the PRRs.

1.3.1 Introduction to interferon system

Type I interferons (IFN), also known as IFN-αβ, were discovered by Isaacs and Lindenmann in 1957. They are produced in response to viral infection. Type II interferon, IFN-γ is secreted by T cells and natural killer cells (NK cells). Type III IFNs have been recently described and consist of IFN-λ1, -λ2 and λ3 that are inducible by viral infection and produce an antiviral state, similar to type I IFN (Kotenko *et al.*, 2001); they are also known as IL-29, IL-28A and IL-28B (Sheppard *et al.*, 2003). The production of IFN is stimulated by the recognition of pathogen associated molecular patterns (PAMPs) by cellular pattern

recognition receptors (PRRs); these receptors activate signal transduction pathways resulting in the production of IFNs. There are many molecules displayed or produced by pathogenic organisms which are considered to be PAMPs, and lead to the induction of IFN. The IFN then acts through cellular receptors, via a signal transduction pathway to induce the transcription of numerous IFN-stimulated genes (ISG)s which collectively produce the antiviral state. The ISGs act in a variety of ways to inhibit viral replication and, ultimately, prevent disease (Reviewed by Randall and Goodbourn, 2008). This review of the interaction between viruses and IFN-α/β system will pay particular attention to RNA viruses (although DNA virus examples of certain features will be used where appropriate), describe the detection of viral PAMPs, in particular double stranded RNA (dsRNA), the pathways involved in IFN induction and transcription of the ISGs, the nature of the antiviral state and various strategies employed by RNA viruses to avoid detection and inhibit those cellular pathways.

1.3.2 Detection of viral PAMPs

RNA viruses induce an IFN response, largely by their production of dsRNA. Studies on the induction of IFN by synthetic polynucleotide complexes revealed polyinosinic polycytidylic acid (poly I:C), a dsRNA homologue, to be a potent inducer of IFN (Field *et al.*, 1967). It was later demonstrated by work on defective interfering particles of vesicular stomatitis virus (VSV), in which a single molecule of dsRNA was presumed to be formed, that the dsRNA induced IFN, whereas conventional particles containing ssRNA did not (Marcus and Sekellick, 1977). This work indicated that dsRNA, which is produced by viruses during their replication, was the most important viral inducer of IFN (Marcus, 1983).

Viral dsRNA may be detected via endosomal delivery or within the cell cytoplasm. The toll-like receptors (TLRs) are a family PRRs; TLR3 has been demonstrated to be a receptor for dsRNA, and that its activation results in the induction of IFN (Alexopoulou *et al.*, 2001). Before stimulation, TLR3 is localised to the endoplasmic reticulum, but then moves to the endosome containing dsRNA. The recognition of dsRNA requires an acidic pH within the

endosome (de Bouteiller *et al.*, 2005). TLR3 has also been identified on the cell surface of a human fibroblast cell line, enabling the extracellular detection of dsRNA and IFN induction (Matsumoto *et al.*, 2002). As well as IFN induction, dendritic cells that have phagocytosed other virus infected cells contain TLR3 that is able to detect viral dsRNA; the cells are activated and induce a cytotoxic T cell response (Schulz *et al.*, 2005). Studies of TLR3 knockout mice have revealed that it is an essential part of the mouse's ability to resist infection with some viruses e.g. murine cytomegalovirus (although a DNA virus, it is assumed to produce dsRNA as a result of bidirectional transcription) (Tabeta *et al.*, 2004) whereas it appears not to have the same role in other viral infections e.g. lymphocytic choriomeningitis (LCMV) virus, VSV and reovirus (Edelmann *et al.*, 2004). These studies showed that although TLR3 is an important receptor for viral dsRNA in some viral infections, there must still be other systems operating in the induction of IFN by other viruses, which will be described later.

TLR3 induces IFN via a signal transduction pathway. After detection of dsRNA, TLR3 undergoes a tyrosine phosphorylation, accompanied by dimerization (Sarkar *et al.*, 2004). The phosphorylated TLR3 then recruits toll-interleukin (IL)-1 domain adaptor inducing IFN-β (TRIF) which results in the activation of two cascades that result in IFN induction (Hoebe *et al.*, 2003; Jiang *et al.*, 2004).

The first cascade, the NF-κB branch, begins with the association of TRIF with tumour necrosis factor receptor-associated factor 6 (TRAF6) the receptor-interacting protein 1 (RIP1) (Sato *et al.*, 2003; Meylan *et al.*, 2004; Cusson-Hermance *et al.*, 2005). TRAF6 functions with the ubiquitin conjugating enzyme Ubc13 to catalyze the synthesis of unique lysine-linked polyubiquitin chains (Deng *et al.*, 2000) which bind to TAK1-binding proteins 2 and 3 (TAB 2 and 3) and activate transforming growth factor β-activated kinase 1 (TAK1). Polyubiquitinated RIP1 is able to recruit IKK through the binding between the polyubiquitin chains and the NF-κB essential modifier (NEMO), which is a regulatory subunit of the IKK complex (Ea *et al.*, 2006). As a result of the creation of a TRIF-RIP1-TRAF6-TAB-TAK1 complex, TAK1 phosphorylates and activates IKK (Wang *et al.*, 2001), leading to the phosphorylation of IκB, its

degradation and release of NF-κB from its bound, inactive form (Baeuerle and Baltimore, 1988) which migrates to the nucleus (Randall and Goodbourn, 2008).

The second branch, after TRIF, is the IFN regulatory factor 3 (IRF3) pathway. As well as TRAF6, TRIF also recruits TRAF3, and they have distinct functions (Häcker *et al.*, 2006). The modulator of TRAF3 signalling is TANK (Li *et al.*, 2002), which interacts with TANK-binding kinase 1 (TBK1) and IκB kinase (IKK) (Pomerantz and Baltimore, 1999) which are essential parts of the IRF3 signalling pathway (Fitzgerald *et al.*, 2003) as they can phosphorylate the C-terminal part of IRF3 and are important in activation (Zhang *et al.*, 2009). Two recently described adaptor proteins have also been described as having a role in IRF3 activation, they are NK-κB-activating kinase-associated protein 1 (NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD) (Sasai *et al.*, 2004; Ryzhakov and Randow, 2007).

TLR3 is not involved in the detection of all RNA viruses, as not all viruses are detected intraendosomally. The detection of intracytoplasmic dsRNA which leads to IFN induction is via two cytoplasmic RNA helicases. Retinoic acid inducible gene I (RIG-I) was identified as an essential regulator for dsRNA induced signalling in 2004 (Yoneyama *et al.*, 2004) and the related protein melanoma differentiation-associated gene 5 (MDA-5) was also first described in 2004 (Andrejeva *et al.*, 2004). Both RIG-I and MDA-5 have been shown to respond to the synthetic dsRNA analogue poly I:C (Rothenfusser *et al.*, 2005; Yoneyama *et al.*, 2005; Gitlin *et al.*, 2006) although more recent work has shown that RIG-I is selectively activated by viral RNA transcripts and MDA-5 by poly I:C (Kato *et al.*, 2006). It has been shown that the ligand which RIG-I recognises is the 5' triphosphate group, which is present in many viral RNAs, and is therefore recognized as non-self (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). The presence of cap structures or a VPg on the viral RNA means there is no 5' triphosphate group to be detected. This is one reason that different viruses are detected preferentially by each of the two helicases e.g. MDA-5 detects picornaviruses whereas RIG-I detects influenza viruses and Japanese encephalitis virus (Kato *et al.*, 2006). Another difference in the nature of dsRNA molecules detected by RIG-I and MDA-5 are lengths of dsRNA, with

MDA-5 detecting long dsRNA, >1 kb, whereas RIG-I has been shown to detect short (<1 kb) RNAs (Kato *et al.*, 2008). RIG-I and MDA-5 have a similar structure, they have C-terminal domains (CTD), a DExD/H-box helicase and an effector domain called the caspase recruitment domain (CARD). The CTDs of both RIG-I and MDA-5 are responsible for the recognition of dsRNA (Cui *et al.*, 2008; Li *et al.*, 2009). The CARD is the effector domain, after activation it interacts with a mitochondrial adaptor protein which has been given several names: CARD adaptor IFN-β (Cardif) (Meylan *et al.*, 2005), virus-induced signalling adaptor (VISA) (Xu *et al.*, 2005), mitochondrial antiviral signalling protein (MAVS) (Seth *et al.*, 2005) or IFN-β promoter stimulator protein (IPS-1) (Kawai *et al.*, 2005). Both RIG-I and MDA-5 use this same adaptor protein in their signalling pathways (Kumar *et al.*, 2006). During its activation, RIG-I undergoes ubiquitination by tripartite motif-containing 25 (TRIM25) which is essential for its IFN induction capability, although this is not true of MDA-5 (Gack *et al.*, 2007). Another difference between the two helicases is in their regulation in the cell; MDA-5 in under the regulation of dihydroxyacetone kinase (DAK), of previously unknown function, whereas RIG-I is not (Diao *et al.*, 2007).

A third helicase, related to RIG-I and MDA-5 is LGP2; unlike the other two, LGP2 has no CARD domain and was initially described as a negative feedback regulator of IFN induction, by sequestering dsRNA (Rothenfusser *et al.*, 2005; Yoneyama *et al.*, 2005). It was subsequently shown that LGP2$^{-/-}$ mice showed markedly different antiviral responses, being resistant to VSV, but having a deficient IFN response to encephalomyocarditis virus (Venkataraman *et al.*, 2007).Recent work has shown that the CTD of LGP2 is able to bind to the blunt ends of dsRNA, and the authors speculate that RIG-I binds to the 5' triphosphate groups in a similar way, and that LGP2 may regulate RIG-I through a direct interaction, rather than by competition for the ligand (Li *et al.*, 2009).

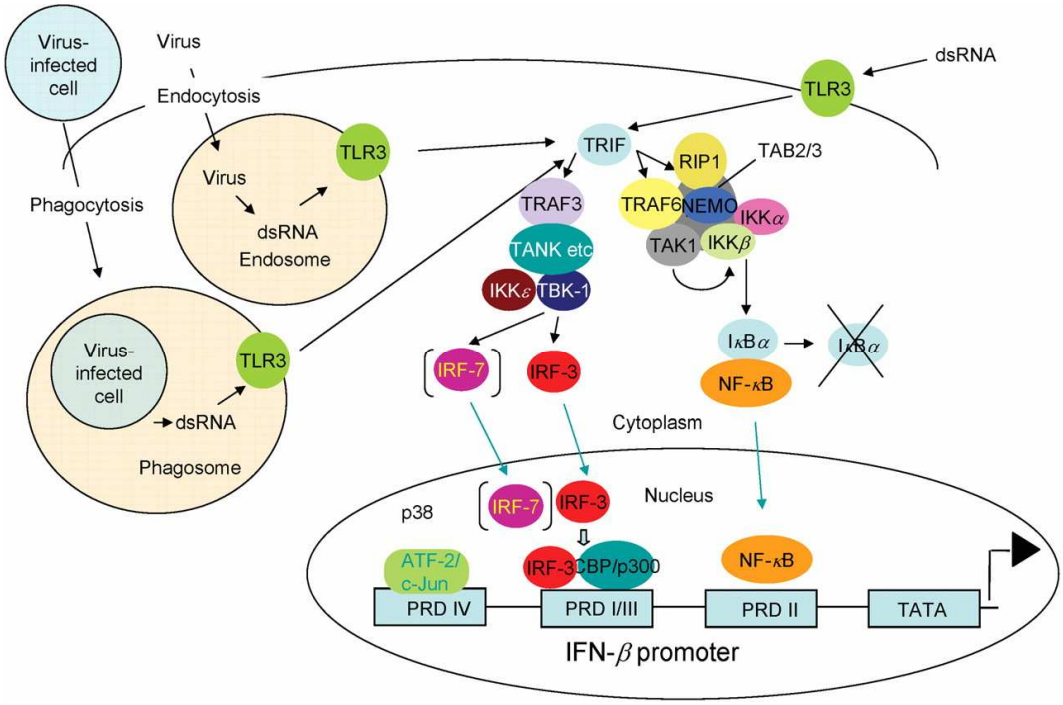


Figure 1.5 TLR3 signal transduction pathway; from Randall and Goodbourn, 2008

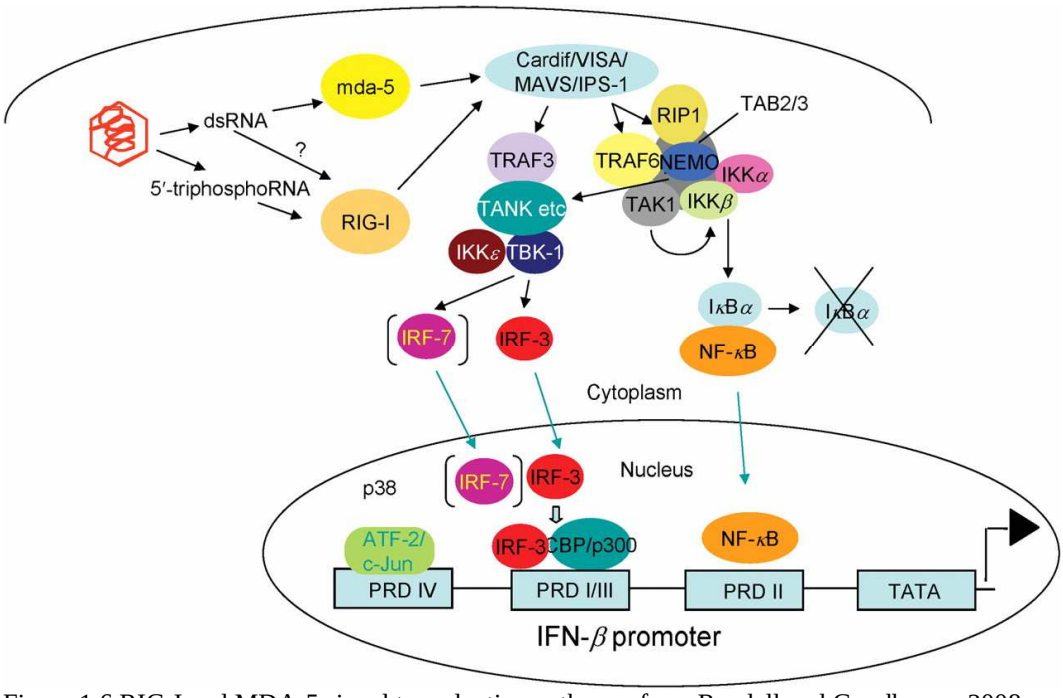


Figure 1.6 RIG-I and MDA-5 signal transduction pathway; from Randall and Goodbourn, 2008

1.3.3 IFN induction pathways

IFN-β is induced by the activation of IRF3 and NF-κB. IRF3 is activated by the phosphorylation, by IKK and/or TBK1, of the Ser/Thr residues in the C-terminal domain, resulting in its dimerization, cytoplasm-to-nucleus translocation, interaction with co-activators CBP/p300 and association with DNA (Panne *et*

al., 2007; Dragan *et al.*, 2007; Sato *et al.*, 1998; Lin *et al.*, 1998). NF-κB is maintained in its inactive form by being bound to IκB (Baeuerle and Baltimore, 1988), which is ubiquitinated and degraded, allowing free NF-κB to translocate to the nucleus (Chen, 2005; Wullaert *et al.*, 2006; Hayden and Ghosh, 2004). The transcriptional activation of the IFN-β gene is dependent on the assembly of a complex consisting of the transcription factors ATF-2/c-Jun, IRF3, NF-κB the high-mobility group chromatin-associated protein (Y) (HMGI(Y)); this complex is called the enhanceosome (Maniatis *et al.*, 1998; reviewed by Merika and Thanos, 2001). These factors cooperate to bind to a DNA site and activate the expression of the IFN-β gene (Panne *et al.*, 2004). Although the enhanceosome is formed, the consensus view is that it is only the binding of IRF3 and/or IRF7 which is truly indispensable for IFN-β induction and that the other elements may not be necessary in certain cell types (Randall and Goodbourn, 2008). During a viral infection, the production of IFN-β leads to the production of other transcription factors that may have positive or negative regulatory effects. IRF1 and IRF2 have been identified as an activator and repressor of IFN genes respectively (Kawakami *et al.*, 1995). Low levels of IFN-β can act in an autocrine or paracrine way to stimulate the expression of IRF7 and IRF5 which act as a positive feedback and amplify the IFN response, an example of this is the role of fibroblast IFN-β in the induction of IFNs in peripheral tissues (Marie *et al.*, 1998; Sato *et al.*, 1998; Erlandsson *et al.*, 1998; Paun and Pitha, 2007). In fact, IRF7 can be considered the master regulator of IFNαβ expression as $IRF7^{-/-}$ are unable to express IFNαβ genes at all upon viral infection (Honda *et al.*, 2005).

1.3.4 Signalling responses to IFN

The cell surface receptor for IFN was first described by Friedman in 1967, but it was not until 1990 that the first IFN receptor identified was cloned (Uze *et al.*, 1990). When a second receptor molecule was cloned in 1994 (Novick *et al.*, 1994) the genes were designated *IFNAR1* and *IFNAR2* (reviewed by de Weerd *et al.*, 2007). Each subunit interacts with a member of the Janus activated kinase (JAK) family; IFNAR1 is associated with tyrosine kinase 2 (Tyk2) and IFNAR2 associates with JAK1 (Darnell *et al.*, 1994; Ihle, 1995). A ligand-dependant rearrangement and dimerization leads to activation of the JAKs (Platanias,

2005). There are many genes induced by IFNs (Der *et al.*, 1998), but the most important antiviral signalling pathways activated are the signal transducer and activator of transcription (STAT) pathways, which were first described in the 1990s (Schindler *et al.*, 1992; Fu *et al.*, 1992; Silvennoinen *et al.*, 1993). The ligand-induced dimerization of the receptor and phosphorylation of tyrosine sites on the cytoplasmic tail of the receptor creates a docking site for the SH2 domains of the STATs (Greenlund *et al.*, 1994; Darnell, 1997). Tyk2 then phosphorylates STAT2, and JAK1 phosphorylates STAT1, and the two phosphorylated STATs form a heterodimer (Randall and Goodbourn, 2008). After stimulation of the pathway, STAT2 which normally is constantly shuttling between the nucleus and cytoplasm, is only imported into the nucleus; this results in the STAT1-STAT2 heterodimer accumulating in the nucleus (Banninger and Reich, 2004; Frahm *et al.*, 2005). The heterodimer then interacts with IRF9 in the nucleus, leading to the creation of IFN-stimulated gene factor-3 (ISGF3) heterotrimer which binds to the IFN-stimulated response element (ISRE) (Tang *et al.*, 2007). The complex is activated by acetylation of all three components of the complex by CREB-binding protein (CBP), previously recruited by the IFNR2. The ISGF3 complex then regulates antiviral gene expression (Tang *et al.*, 2007).

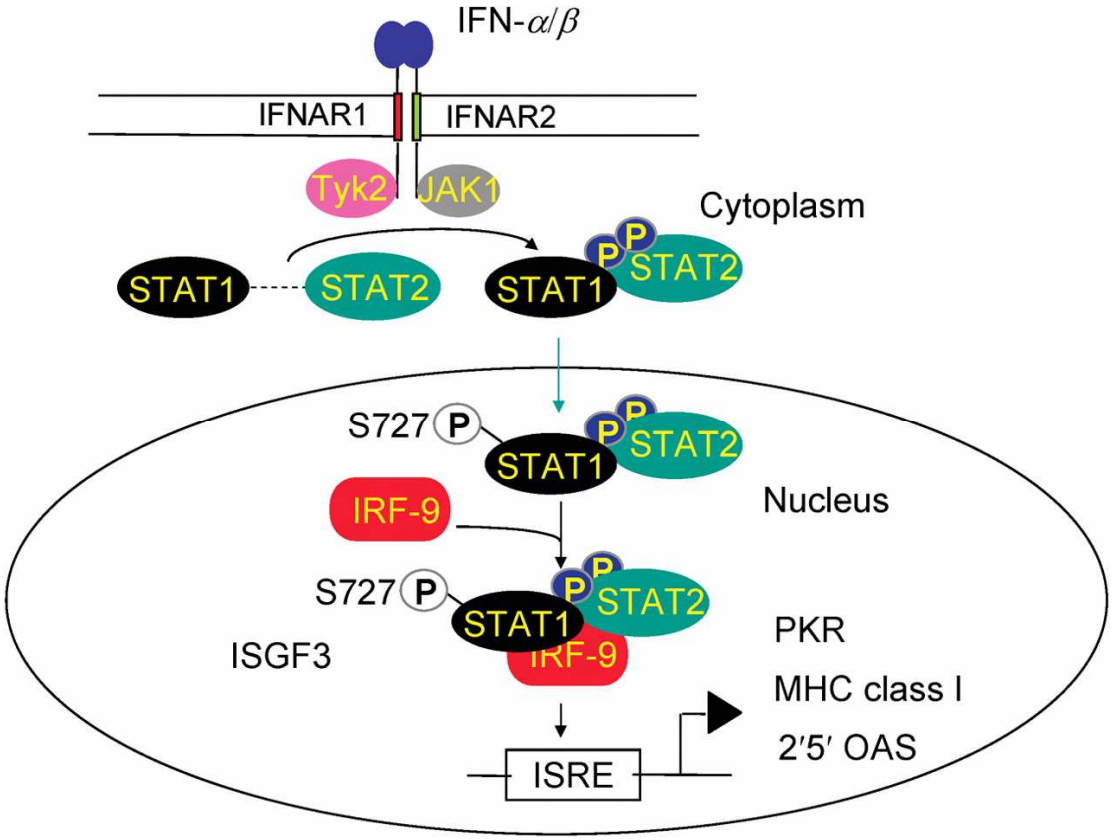


Figure 1.7 IFN effector transduction pathway; from Randall and Goodbourn, 2008

1.3.5 IFN effector mechanisms

1.3.5.1 Antiviral state

As a result of the action of IFN the genes that are expressed result in the cell going into what is known as the antiviral state. Several hundred genes have been identified, but there are several important genes that have been studied in depth and their actions elucidated.

1.3.5.2 PKR

dsRNA-dependant protein kinase (PKR) is an IFN induced molecule first described by Metz and Esteban in 1972. PKR is a protein with dsRNA-dependant kinase activity but exists in a latent state (Garcia *et al.*, 2006). It is activated after recognising and binding dsRNA (Clemens, 1997); due to structural constraints, it is only able to detect dsRNA molecules of greater length than 30 bp (Manche *et al.*, 1992). After binding dsRNA, PKR undergoes various conformational changes and homodimerises, becoming active in the process. The homodimer is further stabilised by autophosphorylation (Dey *et al.*, 2005). PKR is activated in several other conditions: by TLRs, by growth factors and cytokines and in response to cell stress (Garcia *et al.*, 2006). PKR is a member of a family of kinases called the eukaryotic initiation factor 2- (eIF-2α) specific kinases (de Haro *et al.*, 1996), which inhibit the function of eIF-2α and therefore inhibit protein synthesis (Rhoads, 1993). The ability of PKR to detect long viral dsRNAs and inhibit cellular protein synthesis means that it also inhibits viral protein synthesis. PKR is also able to phosphorylate the NF-κB inhibitor IκB-α, removing the inhibition and therefore promoting the further induction of IFN-β. The actions of PKR on both eIF-2α and NF-κB have also been shown to be mediators in apoptosis (Lee and Esteban, 1994; Gil *et al.*, 1999).

1.3.5.3 2’5’ OAS

The discovery of the 2’5’-oligoadenylate synthetase (2’5’OAS) system and its association with the activation of the latent RNase (RNase L) took place in the late 1970s (Hovanessian *et al.*, 1977; Clemens and Williams, 1978). As one of the many ISG (3), dsRNA stimulation of OAS leads to the generation of short (trimer, tetramer, pentamer) 5’phosphorylated, 2’5’-linked oligoadenylates (2-

5A) from ATP (Kerr and Brown, 1978; Minks *et al.*, 1979). 2-5A is degraded very quickly by 2'phosphodiesterase (2'PDE) and 5'phosphatases (Kubota *et al.*, 2004). The most important function of 2-5A is its activation of RNase L in response to IFN during viral infection, but also as part of cellular RNA stability (Zhou *et al.*, 1993). RNase L cleaves on the 3' side of UN sequences in RNA molecules, particularly UA, UU and UG sequences, but much less so after CA or AC (Wreschner *et al.*, 1981; Floyd-Smith *et al.*, 1981). The RNase L enzyme consists of an ankyrin repeat domain, which constitutes the 2-5A binding site, a kinase-like domain and the RNase domain (Hassel *et al.*, 1993; Tanaka *et al.*, 2004). In the absence of 2-5A, RNase L exists as a monomer which has no ribonuclease activity (Dong and Silverman, 1995) due to the repressing effects in the last three ankyrin-like repeats (Dong and Silverman, 1997). It is thought that 2-5A binding causes a conformational change in RNase L, releasing the inhibitory effects of the ankyrin repeats, forming dimers and unmasking the ribonuclease domain, leading to the ability to cleave RNA (Dong and Silverman, 1997; Dong *et al.*, 2001). The role of the 2'5'OAS/RNase L system in antiviral defences is illustrated by the fact that RNase $L^{-/-}$ mice have an increased susceptibility to infection with the picornaviruses encephalomyocarditis virus (EMCV) and Coxsackievirus (Zhou *et al.*, 1997; Flodström-Tullberg *et al.*, 2005) or the flavivirus West Nile virus (Samuel *et al.*, 2006). Some viruses, however, have evolved mechanism to avoid the antiviral effects of RNase L; for example in reovirus infection, PKR and RNase L are involved in shutoff of cellular translation, which are actually beneficial to the growth of some reovirus strains (Smith *et al.*, 2005). Other aspects of the antiviral effects of the system are that it is able to induce apoptosis of infected cells via a mitochondrial pathway (Zhou *et al.*, 1997; Castelli *et al.*, 1998a;1998b) and that it induces the transcription of other genes that suppress virus replication (Malathi *et al.*, 2005).

1.3.5.4 Mx

Mx was discovered after an inbred strain of mouse was described as being unusually resistant to influenza A virus (Lindenmann, 1962). It was later found that the resistance observed was due to a single gene, the *Mx1* gene (Reeves *et al.*, 1988). It was later discovered that many of the commonly used inbred mouse strains had non-functional Mx (Staeheli *et al.*, 1988). A second Mx gene,

Mx2, was identified which also contained mutations in BALB/c and CBA mice (Staeheli and Sutcliffe, 1988). Mx proteins are GTPases related to dynamins (Staeheli *et al.*, 1993), they are able to form trimers, self-assemble into rings that tabulate lipids and localises to the smooth endoplasmic reticulum (Melen *et al.*, 1992; Accola *et al.*, 2002). The mechanism(s) by which Mx effects its antiviral properties are still being investigated. A physical interaction between Mx and the viral nucleocapsid of Thogoto virus has been demonstrated (Kochs and Haller, 1999) as well as human Mx being able recognise bunyavirus capsid protein and interfere in its role in viral genome replication (Haller *et al.*, 2007).

1.3.5.5 ISG15

The ISG15 gene was first cloned in 1986 from IFN treated human Daudi cells (Blomstrom *et al.*, 1986). It was classified as a ubiquitin homologue and a novel mechanism for response to IFN in 1992 (Loeb and Haas, 1992). Ubiquitin is a molecule that tags proteins and labels them for degradation or modifies their functions (Liu *et al*, 2005). Ubiquitins require a ubiquitin-activating enzyme (E1), a conjugating enzyme (E2) and sometimes a ligase (E3) (Reviewed by Kerscher *et al.*, 2006). It has been shown that ISGylation is similar to ubiquitination in that is has a specific E1-like activating enzyme, UBE1L (Yuan and Krug, 2001). Two E2-like conjugating enzymes have also been discovered, namely UbcH8 (Zhao *et al.*, 2004) and UbcH6 (Takeuchi *et al.*, 2008). These enzymes activate and conjugate ISG15 to specific protein targets and modulate their activity, rather than marked them for degradation as in ubiquitination (Zhao *et al.*, 2004; Harty *et al.*, 2009). Similar to ubiquitin, ISGylation is also reversible e.g. the enzyme UBP43 cleaves only ISG15 (Catic *et al.*, 2007; Malakhov *et al.*, 2002). Many targets for ISGylation have already been identified, including members of the antiviral IFN systems: PKR, MxA, HuP56, RIG-I and STAT1 (Zhao *et al.*, 2005; Giannakopoulous *et al.*, 2005; Takeuchi and Yokosawa, 2008). ISG also enhances the innate immune response by subverting the ubiquitin mediated degradation of IRF3, thereby providing a positive feedback mechanism (Lu *et al.*, 2006). ISG15 is also able to enhance the signalling of NF-κB; by conjugating with protein phosphatase 2C beta, which normally suppresses NF-κB (Takeuchi *et al.*, 2006). In addition to its

ubiquitin-like role, ISG15 is also secreted as a cytokine as a response to IFN, but its exact extracellular functions are unclear (D'Cunha *et al.*, 1996). Its role in antiviral defence mechanisms has been demonstrated by studies which have shown increased susceptibility to various virus infections e.g. influenza, Sindbis and herpes viruses (Lenschow *et al.*, 2007), although the antiviral state induced by VSV and LCMV was not affected in the absence of ISG15 (Osiak *et al.*, 2005). Two other related proteins are ISG54 and ISG56. They inhibit translation by interacting with the eIF3 complex (Guo *et al.*, 2000; Terenzi *et al.*, 2005).

1.3.5.6 PML nuclear bodies

Promyelocytic leukaemia nuclear bodies (PML NBs) are small nuclear sub-structures which have been linked to a variety of cellular processes, including: sensing DNA damage and cellular stress (Dellaire and Bazett-Jones, 2004), apoptosis (Bernardi and Pandolfi, 2003), senescence (Bischof *et al.*, 2002), ubiquitination (Anton *et al.*, 1999) and the IFN response to viral infection (Regad and Chelbi-Alix, 2001). PML NBs have been shown to be induced by IFN (Chelbi-Alix *et al.*, 1995) and to reduce gene expression in the human foamy virus (Regad *et al.*, 2001), and inhibits the replication of influenza virus and VSV (Chelbi-Alix *et al.*, 1998). The absence of PML in mice rendered them more susceptible to LCMV and VSV (Bonilla *et al.*, 2002). In the case of poliovirus, infection leads to PML activation of the p53 gene, which leads to apoptosis (Pampin *et al.*, 2006).

1.3.5.7 Adenosine deaminases (ADARs)

The RNA-specific adenosine deaminase (ADAR) was discovered when an enzyme, able to unwind dsRNA, was also shown to convert adenosine residues to inosine residues (Bass and Weintraub, 1988). In human cells there are two forms of ADAR1 (Patterson and Samuel, 1995), a constitutively expressed 110 kDa protein (ADAR1-S) and an IFN inducible 150 kDa protein (ADAR1-L) (George and Samuel, 1999; Kawakubo and Samuel, 2000). ADAR1-L was shown to be >70 fold more active than ADAR1-S in assays with substrates edited in either the nucleus or cytoplasm, and also with substrate generated

during viral replication (Wong *et al.*, 2003) leading to the suggestion that ADAR was part of the antiviral innate immune system. The A-to-I domination is a form of genetic recoding and is found in multiple sits in viral RNA. Inosine is recognised as guanosine by ribosomes and transcribing polymerases, this leads to A-to-G transitions in the edited strand and therefore to U-to-C transitions in cRNA; A-to-G hypermutation has been observed in the genome choriomeningitis virus for example (Zahn *et al.*, 2007). A-to-I hypermutations have also been observed in measles virus, parainfluenza virus and VSV (Cattaneo, 1994).

1.3.5.8 Viperin

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible) is a cytoplasmic protein, induced by IFN that was first identified in human cytomegalovirus infection (Chin and Cresswell, 2001). It has since been shown to have antiviral activity against hepatitis C virus (Helbig *et al.*, 2005), influenza virus (Wang *et al.*, 2007), HIV (Rivieccio *et al.*, 2006) and alphaviruses (Zhang *et al.*, 2007). Viperin localises to the cytoplasmic faces of the endoplasmic reticulum (Hinson and Cresswell, 2009); it has been shown to inhibit the release of influenza virus from the plasma membrane of cells by interacting with farnesyl diphosphate synthase (FPPS) leading to altered membrane fluidity by affecting the formation of lipid rafts, affecting the release of the virus (Wang *et al.*, 2007).

1.3.5.9 Apoptosis

IFN is also able to induce a cell to undergo apoptosis; if an infected cell is forced into programmed cell death it can help to remove a source of infection to the remainder of the animal. There are various pathways by which IFNs may act to induce apoptosis: activation of the STAT pathway can induce the expression of caspase 1 (Chin *et al.*, 1997), and it has been shown that IFN-γ can induce Fas, which acts through Fas ligand to cause apoptosis (Dai and Krantz, 1999). PKR has been shown to play a role in virus induced apoptosis e.g. VSV (Gaddy and Lyles, 2007). It is the role of PKR in the phosphorylation of eIF-2α that mediates apoptosis (Srivastava *et al.*, 1998; Gil *et al.*, 1999;

Scheuner *et al.*, 2006). dsRNA generated during viral infections can, independent of protein synthesis, induce apoptosis by the activation of caspase 8 (Iordanov *et al.*, 2005). The tumour suppressor gene, p53, is also involved in IFN signalling; IFN induces the transcription of p53 and this response has been observed in virally infected cells (with VSV) leading to apoptosis (Takaoka *et al.*, 2003; Munoz-Fontela *et al.*, 2005). The mechanism by which virus infection and IFN induction leads to upregulation of p53 have been shown to be a physical association between PKR and p53, resulting in a functional interaction (Cuddihy *et al.*, 1999). As has been stated previously, the 2'5'OAS/RNase L system also plays a role in the induction of apoptosis (Castelli *et al.*, 1998).

1.3.5.10 Cell-cycle arrest

IFNs have been shown to arrest cell growth via a STAT mediated induction of cyclin-dependant kinase inhibitor p21 (Chin *et al.*, 1996). For this reason, IFNs have been used as anti-cancers agents (reviewed by Ferrantini *et al.*, 2007). A family of IFN-inducible proteins, the p200 family also affect the cell cycle. HIN-200 is a nuclear protein that regulates cell differentiation through its mediation of the cell cycle (Dawson and Trapani, 1996).

1.3.5.11 Immunomodulation

IFNs play a role in the transition from innate immunity to adaptive immunity; IFN-αβ are able to upregulate of class I major histocompatibility complex (MHC-I) and elements of the antigen-presenting machinery (reviewed by Le Bon and Tough, 2002). IFN-αβ produced by virus infected cells also has a role in the maturation of dendritic cells (Le Bon *et al.*, 2003) and potentiates the clonal expansion and survival of CD8+ T cells responding to a specific antigen (Tough *et al.*, 1996). IFN can also induce the production pf IL-15 which is central to the proliferation and survival of T cells (Boyman *et al.*, 2007) and maintain NK cell populations (Nguyen *et al.*, 2002)

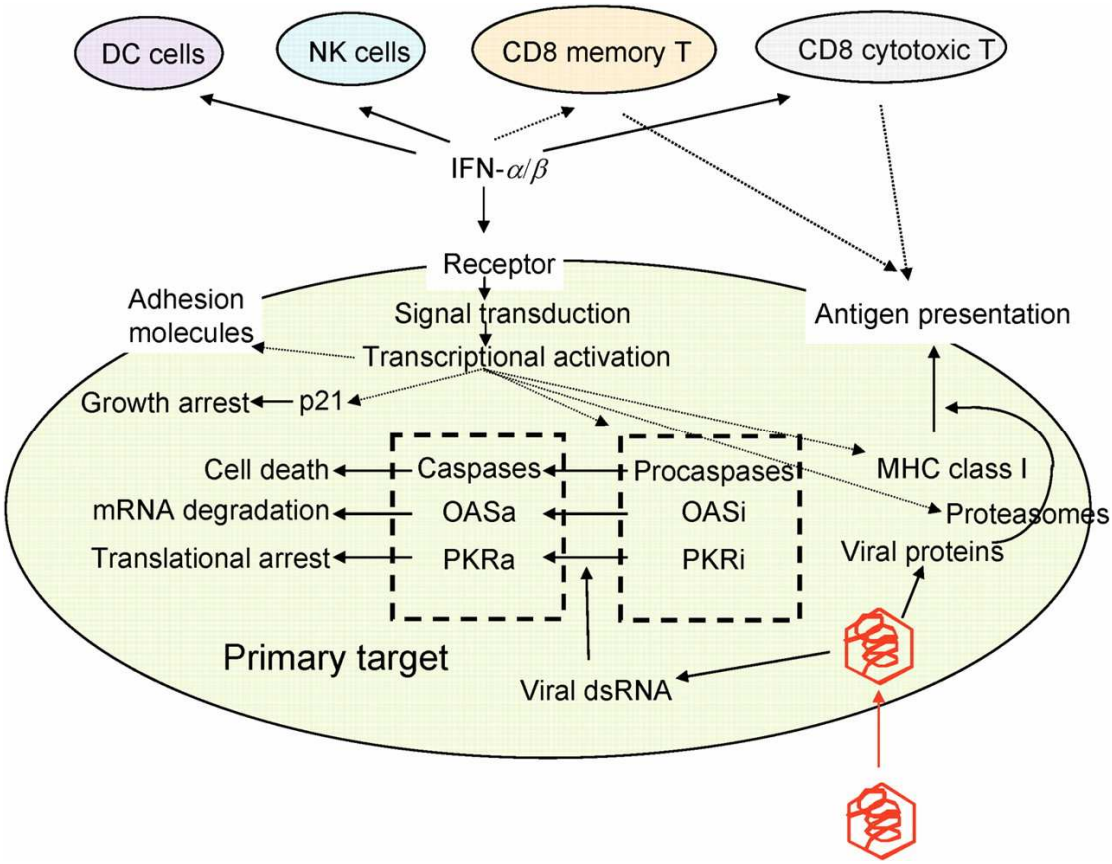


Figure 1.8 IFN effector mechanism pathways; from Randall and Goodbourn, 2008

1.3.6 Viral strategies for avoidance of IFN system

Viruses have developed throughout evolution various means by which to avoid the IFN response. There are five main strategies for circumventing the innate immune response:

i) Interference with host-gene expression

ii) Minimising IFN production by limiting production of PAMPs

iii) Inhibition of IFN signalling

iv) Inhibition of IFN induced enzymes

1.3.6.1 Interference with host-cell gene expression

There are many viruses that are able to inhibit cellular protein expression, often with the result of circumventing the IFN response. Bunyamwera virus (BUNV) non-structural proteins have been shown to target the RNA polymerase II in mammalian cells; this possibly leads to a lytic infection as opposed to the persistent infection seen in BUNV infection of insect cells (Thomas *et al.*,

2004). An advantage to the virus is that inhibiting cellular protein synthesis will also reduce the production of IFN, but the cell may die soon after infection, giving the virus less time to manipulate the cell or establish persistent infections (Randall and Goodbourn, 2008). Table 1.1 shows viruses that avoid the IFN system by global inhibition of host cell gene expression.

Virus	**Mode of action**	**Reference**
Polio virus	Viral proteases cleave: eIF4-G; Poly (A) binding protein TAT box-binding protein	 Etchison *et al.*, 1982 Joachims *et al.*, 1999 Clark *et al.*, 1993
Foot-and-mouth disease virus	Viral proteases cleave: eIF4-G eIF4A	 Kirchweger *et al.*, 1994 Belsham *et al.*, 2000
VSV	M protein inhibits host-cell transcription and translation	Ferran and Lucas-Lenard, 1997
Encephalomyocarditis virus	Disrupts nucleocytoplasmic transport and translation of capped mRNAs.	Porter *et al.*, 2006
Theiler's murine encephalomyelitis virus	L protein interacts with Ran-GTPase and interferes with nucleocytoplasmic trafficking	Delhaye *et al.*, 2004
Influenza A virus	NS1 protein inhibits processing and export of cellular mRNAs	Fortes *et al.*, 1994
Bunyamwera virus	NSs protein inhibits RNAP II to inhibit host cell transcription	Thomas *et al.*, 2004
Rift valley fever virus	NSs protein inhibits host cell transcription by interacting with transcription factor $TF_{II}H$	Bouloy *et al.*, 2001
Eastern equine encephalitis virus	Capsid protein nsP2 inhibits cellular transcription	Aguilar *et al.*, 2007

Table 1.1: Viruses that avoid the IFN system by global inhibition of host cell gene expression (Adapted from Randall and Goodbourn, 2008)

1.3.6.2 Minimizing IFN production

In order to minimise the production of IFN, viruses have evolved replication strategies which minimize the production of PAMPs; they have achieved this by:

i) Controlling virus transcription and translation to minimize the production of PAMPs.

ii) Encapsidating RNA

iii) Protecting the 5' triphosphate group from RIG-I

iv) Integrating their genomes into host chromosomes

v) Hiding PAMPs from cellular receptors

(Randall and Goodbourn, 2008)

As dsRNA is one of the most important PAMPs in viral infections, many viruses have developed means of binding or sequestering it. The reovirus protein σ3 is able to inhibit the activity of PKR by binding dsRNA (Imani and Jacobs, 1988), a similar mechanism has been shown for the core protein σA of the avian reovirus (Martinez-Costas *et al.*, 2000). The porcine group C rotavirus NSP3, which shares homology with dsRNA-binding proteins, also bind dsRNA and inhibit PKR (Langland *et al.*, 1994). Other viral genes that encode proteins that have dsRNA sequestering properties include the E3L gene of vaccinia virus (Chang *et al.*, 1992), the VP35 of Ebola virus (Cardenas *et al.*, 2006), the SM protein of Epstein-Barr virus (Poppers *et al.*, 2003), the pTRS1 of human cytomegalovirus (Hakki and Geballe, 2005), the m142 and m143 proteins of murine cytomegalovirus form a homodimeric complex that is also able to bind dsRNA (Child *et al.*, 2006; Valchanova *et al.*, 2006), the E^{rns} glycoprotein of bovine viral diarrhoea virus (BVDV) has dsRNA-binding activity and RNase activity (Iqbal *et al.*, 2004). The dsRNA binding ability of influenza A virus NS1 protein has also been shown to inhibit the 2'5'OAS/RNase L pathway (Min and Krug,. 2006), prevent the activation of IRF3 (Talon *et al.*, 2000) and is also able to inhibit the action of PKR by binding directly to it (Li *et al.*, 2006). Viruses have developed the ability to avoid detection by directly inhibiting the cytoplasmic PRRs. The V protein of paramyxoviruses inhibits MDA-5 activity (Andrejeva *et al.*, 2004), although RIG-I is unaffected (Childs *et al.*, 2007). The paramyxovirus Sendai virus C protein can also block RIG-I (Strahle *et al.*,

2007). Influenza A virus NS1 protein, however, inhibits RIG-I (Guo *et al.*, 2007) by forming a complex between NS1, RIG-I and Cardif/IPS1 (Mibayashi *et al.*, 2007).

In addition to preventing the detection of dsRNA by TLR3, RIG-I or MDA-5, viruses have also evolved various means to inhibit the signalling cascade emanating from the cellular PRRs. Hepatitis C virus (HCV) NS3/4A is a protease that cleaves the TLR adaptor protein TRIF (Li *et al.*, 2005). Vaccinia virus proteins A52R and A46R are able to target multiple adaptors in the IFN signalling pathway including IRAK2, TRAF6, MyD88 and TRIF leading to a reduction in IFN production (Stack *et al.*, 2005; Harte *et al.*, 2003). Plasmacytoid dendritic cells (pDCs) are able to detect viruses without being directly infected, recognition of ssRNA viruses is through TLR7 after transport of dsRNA replicatory intermediates through the cytoplasm to the lysosome by autophagy. Autophagy is a cellular system for the degradation of proteins in lysosomes, and has been shown also to target intracellular pathogens (Lee *et al.*, 2007; reviewed by Kirkegaard *et al.*, 2004; Schmid *et al.*, 2006). It is also required for the production of IFN by pDCs (Lee *et al.*, 2007). Macroautophagy is induced by phosphorylated eIF2α, a result of the action of PKR and therefore part of the IFN response (Talloczy *et al.*, 2002). Several RNA viruses have hijacked the system for their own advantage though e.g. mouse hepatitis virus (Prentice *et al.*, 2004), poliovirus (Suhy *et al.*, 2000) and equine arteritis virus (Pedersen *et al.*, 1999) are able to assemble their replication complexes at double-membraned vesicles which are reminiscent of autophagosomes (Schmid *et al.*, 2006). The limiting of IFN production is achieved through the Atg5-Atg121 conjugate, a regulator of autophagy, which directly associates with the CARD of RIG-I (Jounai *et al.*, 2007).

Other parts of the transduction cascade are also targeted by viruses, including inactivation of Cardif/VISA/MAVS/IPS-1, inhibition of IRF-3, inhibition of IKK complexes, inhibition of NF-κB and interference with the IFN-β promoter; the strategies for minimizing IFN production are summarized in table 1.2. It is notable that certain viruses e.g. vaccinia virus and HCV, act in more than one

location in the IFN production cascade, as well as other parts of the IFN system to be discussed later.

Virus (protein)	**Mode of action**	**Reference**
Bovine viral diarrhoea virus (E^{rns})	Degradation of dsRNA	Iqbal *et al.*, 2004
Vaccinia virus (E3) Reoviruses (σ3/σA) Rotaviruses (NSP3) Influenza A virus (NS1) Epstein-Barr virus (US11) Human cytomegalovirus (TRS1) Ebola virus (VP35) Murine cytomegalovirus (m142/143)	Sequestration of dsRNA	Chang *et al.*, 1992 Imani and Jacobs, 1988; Martinez-Costas *et al.*, 2000 Langland *et al.*, 1994 Talon *et al.*, 2000 Poppers *et al.*, 2000 Hakki and Geballe, 2005 Cardenas *et al.*, 2006 Child *et al.*, 2006
Hepatitis C virus (NS3/4a) Vaccinia virus	Inhibition of TLR signalling	Li *et al.*, 2005 Harte *et al.*, 2003
Paramyxoviruses (V) Influenza A virus (NS1)	Inhibition of RIG-I / MDA-5	Andrejeva *et al.*, 2004 Guo *et al.*, 2006 Mibayashi *et al.*, 2006
Hepatitis C virus (NS3/4a)	Inactivation of Cardif / VISA / MAVS / IPS-1	Li *et al.*, 2005
Borna disease virus (P) Vaccinia virus (N1)	Interferes with VAK	Unterstab *et al.*, 2005 DiPerna *et al.*, 2004
Rabies virus (P) Hantavirus (G1) Human herpes virus-6 (E1) Rotaviruses (NSP1) Bovine viral diarrhoea virus (N^{pro}) Classical swine fever virus (N^{pro}) Human papillovirus 16 (E6) Herpes simplex virus (ICP0) Bovine herpes virus 1 (bICP0) Thogoto virus (ML) Bovine respiratory syncytial virus Human respiratory syncytial virus SARS corona virus	Inhibition or degradation of IRF-3	Brzozka *et al.*, 2005 Alff *et al.*, 2006 Jaworska *et al.*, 2007 Graff *et al.*, 2007 Hilton *et al.*, 2006 Bauhofer *et al.*, 2005 Ronco *et al.*, 1998 Lin *et al.*, 2004 Saira *et al.*, 2009 Hagmaier *et al.*, 2004 Bossert *et al.*, 2003 Spann *et al.*, 2005 Kopecky-Bromberg *et al.*, 2007

Human papillovirus (E7) Hepatitis C virus (core) Vaccinia virus Adenovirus (E3)	Interferes with the IKK complex	Spikovsky *et al.*, 2002 Joo *et al.*, 2005 DiPerna *et al.*, 2004 Friedman and Horwitz, 2002
African swine fever virus (A238L) Vaccinia virus (N1) Myxoma virus (MNF)	Inhibition of NF-κB	Powell *et al.*, 1996 Shisler and Jin, 2004 Camus-Bouclainville, 2004
Human herpes virus 8 (IRF orthologues)	Interfere with IFN-β promoter activation	Offermann, 2007
Semliki Forest virus (NSP2)	Inhibits IFN production, uncertain mechanism	Breakwell *et al.*, 2007

Table 1.2: Viruses that inhibit IFN production in infected cells (Adapted from Randall and Goodbourn, 2008).

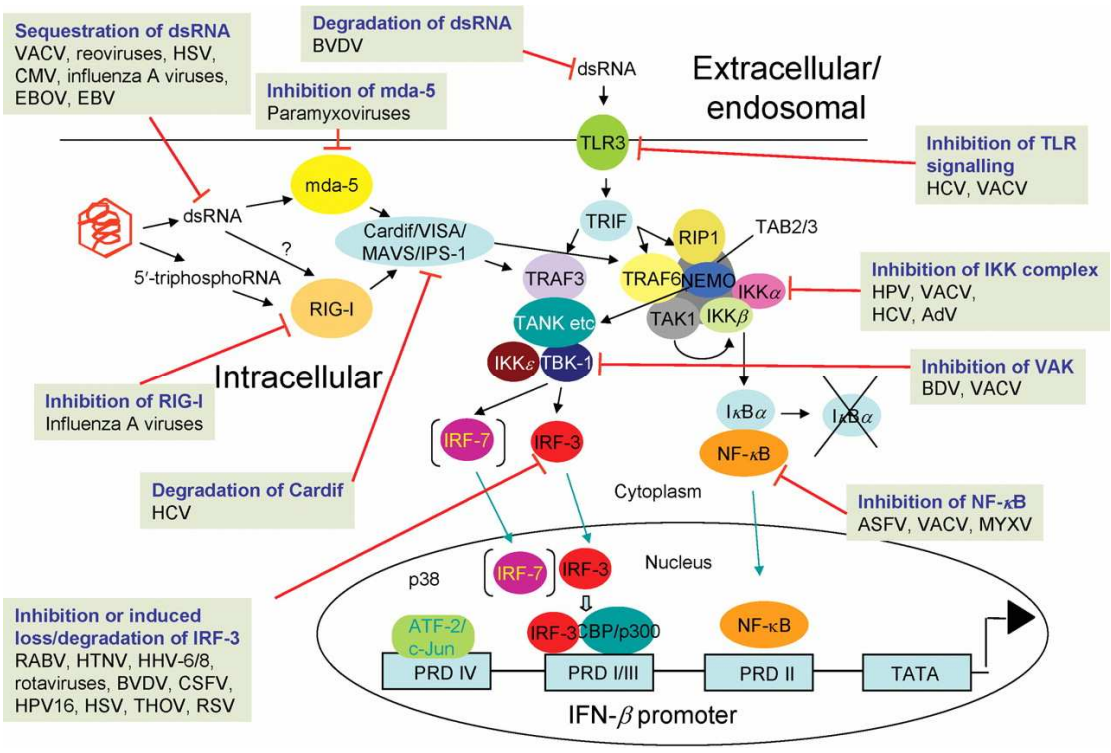


Figure 1.9 Viral strategies for evasion of detection and induction of IFN-β; from Randall and Goodbourn, 2008

1.3.6.3 Inhibition of IFN signalling

Viruses have also evolved mechanisms to counter the IFN signalling cascades, reducing the production of antiviral products and preventing the generation of the antiviral state. Viruses from many families and genera have evolved these mechanisms, and many viruses are able to interfere with multiple parts of the signalling cascade. Examples of mechanisms of counteracting IFN signalling, and the viruses that produce them, include: sequestration of IFN, by orthopoxvirus IFN-α/β binding proteins for example (Alcami *et al.*, 2000; Colamonici *et al.*, 1995); down regulation of IFN receptors by human herpes virus 8 (Li *et al.*, 2007); interference with IFN receptor activity by the NS5 protein of Japanese encephalitis virus (Lin *et al.*, 2004, 2006). The STATs are often the targets of viral evasion strategies: parainfluenza 5 targets STAT1 for degradation (Horvath, 2004), but type 2 targets STAT2 (Ulane and Horvath, 2002); mumps virus targets STAT1 and STAT3 (Ulane *et al.*, 2003; Kubota *et al.*, 2005); the V and P proteins of Nipah and Hendra viruses sequester STAT1 and STAT2 (Rodriguez *et al.*, 2002; Rodriguez, 2003); rabies virus protein P forms an inactive complex with STAT1 and STAT2 (Brzozka *et al.*, 2006); HCV core protein prevents the phosphorylation of STAT1 (Lin *et al.*, 2006); SARS coronavirus can inhibit the nuclear transport of STATs. IRF-9 is another target, the E7 protein of human papillomavirus interacts with IRF-9 (Barnard and McMillan, 1999; Barnard *et al.*, 2000). Table 1.3 summarizes the viruses and mechanisms that inhibit IFN signalling.

Virus	**Mode of action**	**Reference**
Vaccinia virus	Sequestration of IFN	Alcami *et al.*, 2000
Human herpes virus 8	Downregulation of IFN receptor	Li *et al.*, 2007
Japanese encephalitis virus	Interference with JAK kinases	Lin *et al.*, 2004
Dengue virus		Ho *et al.*, 2005
Langat virus		Best *et al.*, 2005
Human cytomegalovirus		Miller *et al.*, 1998
Murine polyoma virus		Weihua *et al.*, 1998
Measles virus		Yokota *et al.*, 2003
Mumps virus		Kubota *et al.*, 2002
Herpes simplex virus		Chee and Roizman, 2004
Human papillomavirus 18		Li *et al.*, 1999

Parainfluenza virus 2	Degradation / loss of STAT	Ulane and Horvath, 2002
Parainfluenza virus 5		Ulane and Horvath, 2002
Mumps virus		Kubota *et al.*, 2001
Newcastle disease virus		Huang *et al.*, 2003
Respiratory syncytial virus		Ramaswamy *et al.*, 2004
Sendai virus		Garcin *et al.*, 2002
Herpes simplex virus		Chee and Roizman, 2004
Nippah virus	Sequestration of STATs or altering phosphorylation of STATs	Rodriguez *et al.*, 2002
Hendra virus		Rodriguez *et al.*, 2003
Measles virus		Palosaari *et al.*, 2003
Rinderpest virus		Nanda and Baron, 2006
Hepatitis C virus		Lin *et al.*, 2006
Human cytomegalovirus		Paulus *et al.*, 2006
Rabies virus		Brzozka *et al.*, 2006
Sendai virus		Garcin *et al.*, 1999, 2000
Adenovirus		Look *et al.*, 1998
Hepatitis B virus		Christen *et al.*, 2007
Vaccinia virus		Najarro *et al.*, 2001
Mapuera virus		Hagmaier *et al.*, 2007
Ebola virus	Inhibition of STAT trafficking	Reid *et al.*, 2006
SARS corona virus		Frieman *et al.*, 2007
Human papilloma virus 16	Interference with IRF-9	Barnard and McMillan, 1999
Herpes simplex virus		Yokota *et al.*, 2004
Hepatitis C virus		Bode *et al.*, 2003
		Duong *et al.*, 2006
Hepatitis B virus		Christen *et al.*, 2007
		Fernandez *et al.*, 2003
Human herpes virus 8		Rezaee *et al.*, 2006
Adenovirus		Leonard and Sen
SARS corona virus		Kopecky-Bromberg *et al.*, 2007
Dengue virus		Munoz-Jordan *et al.*, 2003
Herpes simplex virus		Chee and Roizman, 2004

Table 1.3: Viruses that inhibit IFN signalling (Adapted from Randall and Goodbourn, 2008)

1.3.6.4 Inhibition of IFN induced enzymes

The interferon stimulated genes that give rise to the antiviral state are also targets for viruses to circumvent the IFN response. There are examples of viral mechanisms of avoiding the effects of PKR, 2'5'-OAS, ISG 15 and PML nuclear bodies. The sequestering of dsRNA is not only effective in preventing the induction of IFN, as has been described, but also prevents the activation of PKR, 2'5'-OAS and ADAR. Apart from dsRNA, PKR also has dsRNA independent cellular activators, PKR associated activator (PACT) in humans (Patel and Sen, 1998), and the murine homologue RAX (Ito *et al.*, 1999). The presence of PKR in the cell as part of a stress response may be the reason that viruses have evolved mechanisms against PKR specifically, mechanisms include: RNA inhibitors, viral RNAs that bind to PKR in competition with dsRNA; direct interaction of a viral protein with PKR; eIF2α homologues that act as pseudo-substrate competitive inhibitors; degradation of PKR and eIF2α phosphatases. Table 1.4 summarizes the viral anti-PKR mechanisms.

Virus (protein)	**Mode of action**	**Reference**
Hepatitis C virus (RNA) Human immunodeficiency virus	RNA inhibitors	Vyas *et al.*, 2003 Gatignol *et al.*, 1991
Hepatitis C virus (NS5A) Vaccinia virus (E3L) Human herpes virus 8 (vIRF-2) Influenza viruses (p58)	PKR interaction / binding	Taylor *et al.*, 1999 Romano *et al.*, 1998 Burysek and Pitha, 2001 Tan *et al.*, 1998; Lee *et al.*, 1992
Vaccinia virus (K3L) Human immunodeficiency virus (tat)	eIF2α homologues	Beattie *et al.*, 1991 Endo-Munoz *et al.*, 2005
Poliovirus	PKR degradation	Black *et al.*, 1993
Herpes simplex virus ($_{\gamma 1}$34.5)	eIF2α phophatase	Chou *et al.*, 1995

Table 1.4: Viral anti-PKR mechanisms (Adapted from Langland *et al.*, 2006)

As has been stated, the action of 2'5'-OAS is reduced by the sequestration of dsRNA, but some viruses have evolved a specific anti-OAS strategy. Human

immunodeficiency virus 1 (HIV-1) and encephalomyocarditis virus (EMCV) induce the production of RNase L inhibitor (RLI) (Martinand *et al.*, 1998; 1999). Inhibition of ISG15 has been shown influenza B NS1 protein by preventing its interaction with the El ligase (Yuan and Krug, 2001). The adenovirus E4orf3 protein has been shown to interact with PML nuclear bodies and induce a morphological change (Doucas *et al.*, 1996).

1.4 RNA stability

The genomes of positive-sense RNA viruses are equivalent to cellular mRNAs in that they are released into the cell to be translated by ribosomes. In the cell, control of mRNA degradation is an important factor in the regulation of gene expression and many of the enzymes and pathways have been described over recent years (reviewed by Garneau *et al*., 2007). Cellular mRNAs have 7-methylguanosine caps at their 5' ends, and a poly(A) tail at their 3' ends. In the majority of mRNAs, the degradation process begins with shortening of the poly(A) tail followed by either degradation in a 3'-5' direction by a 10-12 subunit complex of exonucleases called the exosome; or the 5' cap is removed and the RNA degraded by 5'-3' exoribonucleases, such as Xrn1 (Houseley *et al*., 2006; Garneau *et al*., 2007). The first step, deadenylation, is carried out by the nucleases PAN2 and CCR4; PAN2 initiates deadenylation followed by CCR4-mediated shortening of the poly(A) tail (Yamashita *et al*., 2005). Structures known as P bodies play a role in mRNA control, they are sites where the fates of mRNA molecules in determined (Eulalio *et al*., 2007). These granular structures contain factors associated with translation initiation (Ferraiuolo *et al*., 2005), deadenylation (Cougot *et al*., 2004), decapping (Sheth and Parker, 2003), decay by 5'-3' exonucleases (Bashkirov *et al*., 1997) and nonsense-mediated decay (NMD) (Sheth and Parker, 2006). P bodies are dynamic and represent only active mRNA decay sites; they disappear when not active (Cougot *et al*., 2004). Endonucleases cleave RNA molecules to produce multiple fragments which are then susceptible to exonucleases (Garneau *et al*., 2007). It has been shown that the protein Argonaute 2, part of the RNA-induced silencing complex (RISC), cleaves RNA as part of the short interfering RNA (siRNA) mechanism (Liu *et al*., 2004). These mechanisms also make up a system of mRNA surveillance by detecting aberrant RNAs. Nonsense-mediated decay detects premature termination codons, non-stop decay degrades mRNAs lacking a stop codon and no-go decay detects stalled ribosomes on an mRNA (Garneau *et al*., 2007). There are various mRNA stability elements that control mRNA decay. One of the most important is the AU-rich element (ARE), often found in the 3' untranslated region of the molecule. The AREs are able to recruit the mRNA-decay machinery through various ARE-binding proteins such as AU-rich binding factor-1 (AUF1), tristetraprolin (TTP) and KH splicing

regulatory protein (KSRP) that interact with various decay factors (Garneau *et al*., 2007).

RNA viruses that deliver their genomes into cells must be able to avoid degradation by the cellular ribonucleases in order to establish infection in a cell. This may be achieved by shielding the RNA physically from RNA decay machinery, or by modulating the decay machinery (Sokoloski *et al*., 2006). Many RNA viruses have been shown to form membrane-bound vesicles during replication flaviviruses and noroviruses, for example (Uchil and Satchianandam, 2003, Hyde *et al*., 2009). Viruses may also produce ribonucleoproteins (RNPs) which may regulate the RNA decay mechanisms. The 3' UTR of hepatitis C virus (HCV) binds to the cellular HuR protein, conferring protection against exonucleases (Spangberg *et al*., 2000). Some viral RNAs have no cap, so would be vulnerable to degradation by ribonucleases. Poliovirus, which is not capped, produces the protein $2A^{pro}$ which is able to cleave parts of the cap-binding complex and the poly(A)-binding protein (PABP), which plays an important role in initiation of translation in eukaryotic cells (Joachims *et al*., 1999). The 2'5'-OAS/RNAse L system has already been described as an IFN stimulated mechanism in which RNase L degrades viral and cellular RNAs. This system may also play a role in regulation of cellular mRNA activity (Bisbal and Silverman, 2007). There is more to be learned about the interaction of viral RNAs with cellular degradation mechanisms, but these interactions may play a role in viral persistence. One of the examples given above, HCV, is one of the best characterised persistent viruses which has evolved many means of avoiding various parts of the cells defences; there may be similar strategies in many other viruses.

1.5 Persistent infections

Persistent infections are a feature of many viral diseases. In order for a persistent infection to be established, viruses have to adopt a replication strategy that allows them to continuously reside in cells instead of killing them, and to be able to evade the innate and/or adaptive response so as not to be cleared from the body (reviewed by Oldstone, 2006; 2009). Whilst many viruses become persistent through their ability to evade host defences, it must be remembered that non-persistent viruses have developed mechanisms to evade cellular defences too over the course of their evolution.

Hepatitis C virus (HCV) is one of the best characterized persistent infections and will be briefly summarized here. HCV is able to produce a persistent infection by various immune evasion mechanisms. The NS3/4A protein is able to disrupt RIG-I signalling which prevents activation of the IRF-3 or NF-κB arms of the IFN-B induction pathway (Foy *et al*., 2005). HCV proteins are also able to inhibit IFN-α/β signal transduction through the JAK-STAT pathway by blocking the binding of STAT transcription factors to the ISG promoters. This happens through the action of protein phosphatase 2A which produces hypomethylated STAT1 which then associated with PIAS1, an inhibitor of STAT DNA binding (Heim *et al*., 1999; Blindenbacher *et al*., 2003; Duong *et al*., 2004). HCV core protein has also been shown to induce the expression of the suppressor of cytokine signalling -3 (SOCS3) (Bode *et al*., 2003). HCV protein NS5A also has a role in IFN antagonism; it is able to induce the expression of interleukin 8 (IL-8) which is associated with reduced 2'5'OAS activity (Khabar *et al*., 1997). HCV protein E2 can inhibit the kinase activity of PKR and block its inhibitory effect on protein synthesis (Taylor *et al*., 1999). Ribonuclease L, the effector enzyme of the 2'5'OAS pathway, selectively cleaves RNA at UA and UU dinucleotides, but HCV has evolved to have a relative lack of these dinucleotides (Han *et al*., 2004). Another aspect of HCV evolution that may enable it to evade immune system and become persistent is that it exists as a population of closely related yet divergent genomes, known as quasispecies. This genetic variability may play a role in protecting the virus from innate or adaptive immunity (Farci and Purcell, 2000).

The cells of the innate immune system include dendritic cells and natural killer (NK) cells. Dendritic cells act as antigen presenting cells in peripheral tissues; they load antigenic peptides onto major histocompatibility complex (MHC) class I and II molecules and present them to T lymphocytes, thus initiating the adaptive immune response (reviewed by Guermonprez *et al*., 2002). Different populations of dendritic cells express different receptors, so are able to detect different microbes (Kadowaki *et al*., 2001). Plasmacytoid dendritic cells (pDC) produce large amounts of IFN-αβ on viral stimulation and promote the function of NK cells, B lymphocytes, T lymphocytes and myeloid dendritic cells (McKenna *et al*., 2005). As they play such an important role in the interface between innate and adaptive immunity, they are often targets for persistent viruses (Liu *et al*., 2009). In persistent infection of mice with lymphocytic choriomeningitis virus (LCMV), the pDCs lose their ability to secrete high levels of IFN-αβ which in turn, reduces the stimulation of NK cells; as a consequence, mice are less able to mount a response to subsequent infection with murine cytomegalovirus (Zuniga *et al*., 2008). It has also been shown that measles virus and LCMV both inhibit the development and expansion of DCs through the generation of IFN that acts through a STAT2 dependant mechanism which has antiproliferation and antidifferentiation effects on DCs (Hahm *et al*., 2005).

Although some viruses are counteracted predominantly by the innate immune system, complete clearance of the virus from the body often depends on the action of the adaptive immune system e.g. MNV, see above. Viruses may also establish persistent infections by evading the adaptive immune system. Studies on LCMV by Oldstone and colleagues demonstrated that viruses and antibodies circulate as immune complexes (Oldstone and Dixon, 1968). These immune complexes are infectious and may contribute to the maintenance of persistent infections and allow the virus to infect different cell types (Oldstone, 2006). Viruses can also alter the processing and migration of their peptides in antigen presenting cells (Oldstone, 2009). Foot-and-mouth-disease virus (FMDV) infections may be persistent in some of the species it infects. FMDV persists in the pharynx of cattle (Zhang and Alexandersen, 2004), and can be detected in the African buffalo for up to 5 years (Hedger, 1972). The precise mechanism is

still unclear, although it is likely that CD8 T cells activation is suppressed (Alexandersen *et al*., 2002). Table 1.5 shows a selection of known persistent viruses described in the book Persistent Viral Infections, edited by Ahmed and Chen, 1999, with brief notes on the mechanism by which they may become persistent taken from each chapter and the most recent references.

Persistent Virus	**Mechanism of immune evasion / establishment of persistence**	**Author of chapter and other references**
Human immunodeficiency virus		Grovit-Ferbas *et al*.
Human T cell leukaemia virus	Integration of proviral DNA into host genome	Kashanchi *et al*.
Hepatitis B virus	Neonatal tolerance, failure of clearance by T cells; T cell hyporesponsiveness	Chisari and Lai; Billerbeck *et al*., 2007
Hepatitis C virus	Multiple evasion strategies	Walker; Gale and Foy, 2005
Polyomavirus	Not fully understood	Lukacher; Comoli *et al*., 2008
Papillomavirus	Tolerance of viral antigens; inhibition of IRF-3	Frazer and Dunn; Ronco *et al*., 1998
Adenovirus	E3 proteins migrate to membrane, protect infected cells from CTL response, also bind to MHC molecules	Lukashok and Horwitz; Fessler *et al*., 2004
Varicella-zoster virus	Downregulation of MHC class I, upregulation of MHC class II	Arvin; Abendroth and Arvin, 2001
Cytomegalovirus	Downregulation of HLA class I and II; protein US3 delays maturation of MHC I	Soderberg-Naucler *et al*.; Liu *et al*., 2009
Epstein-Barr virus	Modulates cytokine balance and apoptotic signals	Hutt-Fletcher; Ohga *et al*., 2002
Herpesviruses 6,7,8	Binding immunoglobulins, inactivating antigen presenting pathways	Meng and Pellett
Measles virus	V protein targets STAT-2	Schneider-Schaulies *et al*.;

		Ramachandran *et al*., 2008
Visna virus	Integrate genome into host DNA	Cheblounе *et al*.
Feline leukaemia virus	Integrate genome into host DNA	Rohn and Overbaugh
Murine adenovirus	Reduction in MHC class I	Smith and Spindler
Bovine papillomavirus	E2 protein ensure tethering of viral genomes to host chromosomes for persistence of viral episomes	Campo; You *et al*., 2004
Theiler's murine encephalomyelitis virus	Restricted expression of viral genes, infection of microglial cells, suppression of MHC; L protein inhibits transcription of IFN genes.	Tsunoda and Fujinami; Ricour *et al*., 2009
Mouse hepatitis virus	Cytotoxic lymphocyte escape mutations	Stohlman *et al*.; Kim and Perlman, 2003
Borna disease virus	Low productivity by control of viral gene expression; inhibition of NF-κB; P protein interacts with TBK1	Schemmle *et al*.; Planz *et al*., 2009
Lymphocytic choriomeningitis virus	Impairment of CTL response	Buchmeier and Zajac; Wherry *et al*., 2003
Bovine diarrhoea virus	Envelope glycoprotein inhibits IFN expression; inhibits induction of apoptosis	Akkina; Matzener *et al*., 2009; Schweizer and Petehans, 2001

Table 1.5 A selection of persistent viral infection with mechanisms of persistence; adapted from Persistent Viral Infections, edited by Ahmed and Chen, 1999.

Several of the viruses referred to in table 1.5 are herpesviruses. Herpesviruses are large dsDNA viruses; a feature of herpesvirus life cycles is their ability to become latent. Varicella-Zoster virus (VZV) for example establishes latent infections in sensory ganglia; the linear DNA genomes assume a circular conformation and there is no active viral replication until later reactivation, or recrudescence (Mueller *et al*., 2008). Latency is distinguished from persistence as in latency there is no viral replication, whereas in persistent infection viral replication continues, all be it at a low level tolerated by the host.

1.6 Genome-Scale Ordered RNA Structure (GORS)

1.6.1 RNA secondary structures in viruses

Secondary structure in RNA molecules was first described by Fresco *et al.*, who described the presence of helical regions of Watson-Crick base-pairing of adenine-uracil and guanine-cytosine. They described how secondary structure arises from intramolecular interactions, the lateral mobility that allows RNA molecules to bend and form local structures such as hairpins (Fresco *et al.*, 1960). RNA viruses' replication strategies often involve the use of structured RNA elements. These elements may be involved in translation and packaging signals during genome encapsidation.

The internal ribosome entry site (IRES) is found in the 5' untranslated regions (UTRs) of picornaviruses such as poliovirus (Pelletier and Soenberg, 1988) and flaviviruses such as hepatitis C virus (HCV) (Tsukiyama-Kohara *et al.*, 1992) as well as dicistroviruses and lentiviruses; the structural and functional diversity of viral IRESes has recently been reviewed by Balvay *et al* (2009). The IRESes consist of several domains of internal base-pairing and the formation of stem loops which interact with host ribosomes during translation of viral genomes. Some of the best characterised IRES structures are those of picornaviruses, although there is marked divergence between the IRES's across the genera, reviewed by Belsham (2009). There are two major types of picornavirus IRES, type I and II. Type I IRESes have up to 14 stem-loop domains, labelled A to N (Jang and Wimmer, 1990), and type two have 6 stem-loops labelled I to VI (Harber and Wimmer, 1993). Enteroviruses and rhinoviruses have type I IRESes, whereas aphthoviruses and cardioviruses have type II. All picornavirus IRESes have a GNRA motif in their central domains and a polypyrimidine tract 20 to 25 nucleotide upstream of an AUG start codon (Balvay *et al.*, 2009). In aphthovirus, the 3' end of their type II IRESes interacts with the eukaryotic initiation factors to drive translation of the viral genome (Kolupaeva *et al.*, 2003) Another of the best characterised IRES's is that of HCV; its structure and function are reviewed by Lukavsky (2009) but a brief summary will be given here. The HCV IRES contains two major domains, II and III, and a smaller domain IV (Brown *et al.*, 1992; Kieft *et al.*, 1999). The IRES extends

from residue 40 to 372 of the viral genome (Fukushi *et al.*, 1994). The largest domain, domain III, consists of branching hairpin stem-loops made up of basal, middle and upper parts, each of which contains further stem-loops (Brown *et al.*, 1992). The function of the IRES is dependant on its secondary structure as has been demonstrated by mutagenesis studies (Brown *et al.*, 1992; Fukushi *et al.*, 1994). Domain III contains the regions that interact directly with eIF3 and the 40S ribosomal subunit leading to a IRES-40S-eIF3 complex and consequent translation (Kieft *et al.*, 2001).

The presence of structured regions, within the coding region of viruses, that have a role to play in replication have also been described. In poliovirus there is a 61 nucleotide stem-loop, from position 4435 to 4495, which is also well conserved in the *Enterovirus* genus. As it was required for viability it was named a *cis*-acting replication element (CRE) (Goodfellow *et al.*, 2000). Similar structures have been described in enteroviruses and rhinoviruses (Xiang *et al.*, 1995), foot-and-mouth disease virus (Mason *et al.*, 2002) and the Brome mosaic virus (Joost Haasnoot *et al.*., 2002).

Stem-loops may also be found in the 3' region of viral genomes. The terminal 46 bases of the HCV genome were described as a stem-loop structure in 1996 (Kolykhalov *et al.*, 1996) which was shown to be essential for HCV infectivity and replication (Kolykhalov *et al.*, 2000; Yi and Lemon, 2003). HCV has many stem-loops in its coding region (Tuplin *et al.*, 2002) one of which has been identified as a CRE (You *et al.*, 2004). A recent study has also shown that there is a direct interaction between the IRES in the 5' UTR and structured elements in the 3' region of the genome (Romero-Lopez and Berzal-Herranz, 2009).

RNA structures also form packaging signals during encapsidation e.g. HIV (Huthoff and Berkout, 2002).

1.6.2 Bioinformatic prediction of RNA secondary structure

RNA secondary structures can be predicted by use of energy minimization algorithms based on dynamic programming (the development of these techniques is reviewed by Mathews, 2006). The principle of these algorithms is

to predict the most energetically favourable structure compared to other potential structures. The nearest neighbour model for predicting secondary structure, described by Tinoco *et al.* 1973, predicts free energy change at 37°C. An RNA molecule will exist in equilibrium between a structured and unstructured state. The free energy change for a given structure quantifies its stability. The structure with the lowest free energy is the most represented at equilibrium, and therefore the single most likely structure (Mathews and Turner, 2006). The nearest neighbour model is so called as it is dependant on the identity of the motif and adjacent base pairs (Mathews, 2006). Algorithms were later developed to include the non-Watson-Crick pairing of guanine-uracil and to account for loop regions (Mathews *et al.*, 2004). Dynamic programming algorithms (Nussinov *et al.*, 1980) implicitly check all the possible secondary structures, although they don't actually generate them. The first step, known as the fill step, the lowest free energy is determined for each fragment, starting with the shortest then moving to the longer fragments. The longer fragments' folding energies are determined by referring back to the energies of the short fragments that have been stored (Mathews and Turner, 2006). The second step, known as traceback, uses the free energies to determine the exact structure that has the lowest free energy (Mathews, 2006). An algorithm that is also able to detect pseudoknots has also been devised (Rivas and Eddy, 1999). These algorithms may not produce reliably the true RNA secondary structure as in nature RNA molecules may have more than a single conformation and form suboptimal foldings. The prediction of suboptimal foldings was described by Zuker in 1989. Another method, developed by McCaskill (1990), called base-pair partition functions, provides information about the probability of secondary structures. Dynamic programming algorithms have been used in programs such as MFOLD (Zuker, 2003), Pfold (Knudsen and Hein, 1999), RNAalifold (Hofacker *et al.*, 2002) HotKnots (Ren *et al.*, 2005) and RNAz (Gruber *et al.*, 2007). One of the latest algorithms is UNA fold which is an integrated program consisting of folding simulation, hybridization and melting pathways which combines free energy minimization, partition function and stochastic sampling (Markham and Zuker, 2008).

1.6.3 Discovery of GORS

Genome-level ordered RNA structure was first described by Simmonds *et al.* in 2004. This study consisted of large-scale thermodynamic predictions of RNA secondary structures across the genomes of viruses from the families *Picornaviridae* and *Flaviviridae*. The program MFOLD (Zuker *et al*., 1989; 2003) was used to predict the most stable secondary structure of genomic fragments throughout the length of complete genome sequences of four genera of *Picornaviridae* (*Enterovirus, Aphthovirus, Teschovirus* and *Hepatovirus*) and four genera of *Flaviviridae* (F*lavivirus, Hepacivirus*, GBV-C-like viruses and *Pestivirus*). The minimum free energy (MFE) of the energetically most stable structure, as predicted by the MFOLD algorithm, of each sequence was compared with the MFE of 50 randomized control sequences. The results were expressed of minimum free energy differences (MFEDs) i.e. the percentage difference between the native sequence and the mean of the 50 randomized controls. Results were also expressed as a Z score. The Z score is the position of the MFE within a distribution of the MFEs of the randomized sequences, expressed as the number of standard deviations from the mean (Workman and Krogh, 1999). Several randomization strategies were used in order to preserve certain non-random elements of the sequences such as dinucleotide frequencies. In viral RNA genomes the dinucleotides CG and UA are underrepresented and CA and UG are overrepresented (Rima and McFerran, 1997). Randomization strategies that preserve non-random elements include:

i) Nucleotide order randomization (NOR): randomization of the sequence order.

ii) Codon order randomization (COS): avoids disruption of sequence order within codons.

iii) Like-codon randomization (CLR): randomization of the order of codons encoding each amino acid but maintaining amino acid sequence.

iv) Like-codon swap (CLS):pairwise exchange of codons specifying each amino acid.

v) Dinucleotide randomization (CDR): randomization of codons with identical first and third bases.

vi) Dinucleotide swap (CDS): pairwise exchange of codons with identical first and third bases.

vii) CDLR: codon composition, order are preserved and dinucleotide frequencies are preserved, combines CLR and CDR.

viii) NDS: retains regional differences in base composition and preserves dinucleotide composition.

ix) NRR: preserves trinucleotide frequencies.

x) NTS: preserves tetranucleotide frequencies.

(Tuplin *et al*., 2002; Simmonds *et al*., 2004).

The result was that the certain genera from *Picornaviridae* and *Flaviviridae* showed areas of predicted structure throughout the lengths of their genomes. Of the *Picornaviridae*, the aphthoviruses and teshcoviruses showed extensive predicted structure whereas hepatoviruses and enteroviruses did not. Of the *Flaviviridae*, HCV and GBV-C showed extensive secondary structure whereas flavivirus and pestivirus did not. This phenomenon was termed genome-scale ordered RNA structure (GORS). Figure 1.10 shows the predicted structure for fragments of the genomes of eight genera.

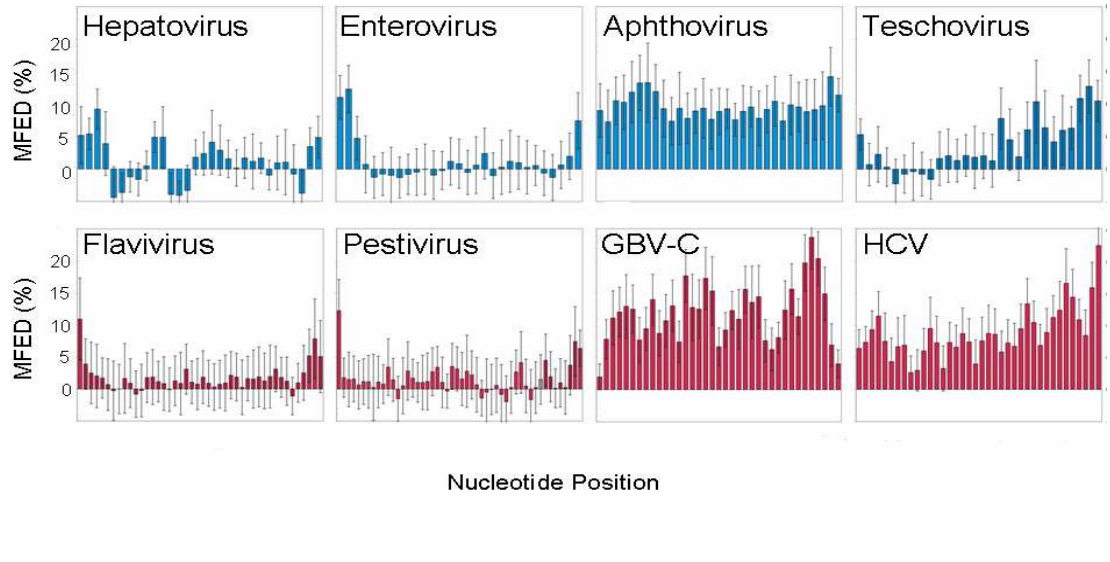


Figure 1.10 The distributions of MFEDs across the genomes of genera of picornaviruses (blue) and flaviviruses (red); each bar represents the mean MFED of a 498 base fragment of the genome. Those genera that possess GORS are *Aphthovirus*, *Teschovirus*, *GBVC-like virus* and *Hepacivirus*. Adapted from Simmonds *et al*., 2004.

1.6.4 Possible roles of GORS in viral persistence

GORS was not considered to be part of a fundamental replication strategy as within virus families, some genera have GORS and others do not, and replication strategies are usually conserved within families. An association was observed, however, between the presence of GORS and the virus's ability to establish persistent infections in their natural hosts. The viruses with the highest MFED scores, HCV, GBV-C-like and the genus *Aphthovirus* are all known to establish persistent infections; see figure 1.11..

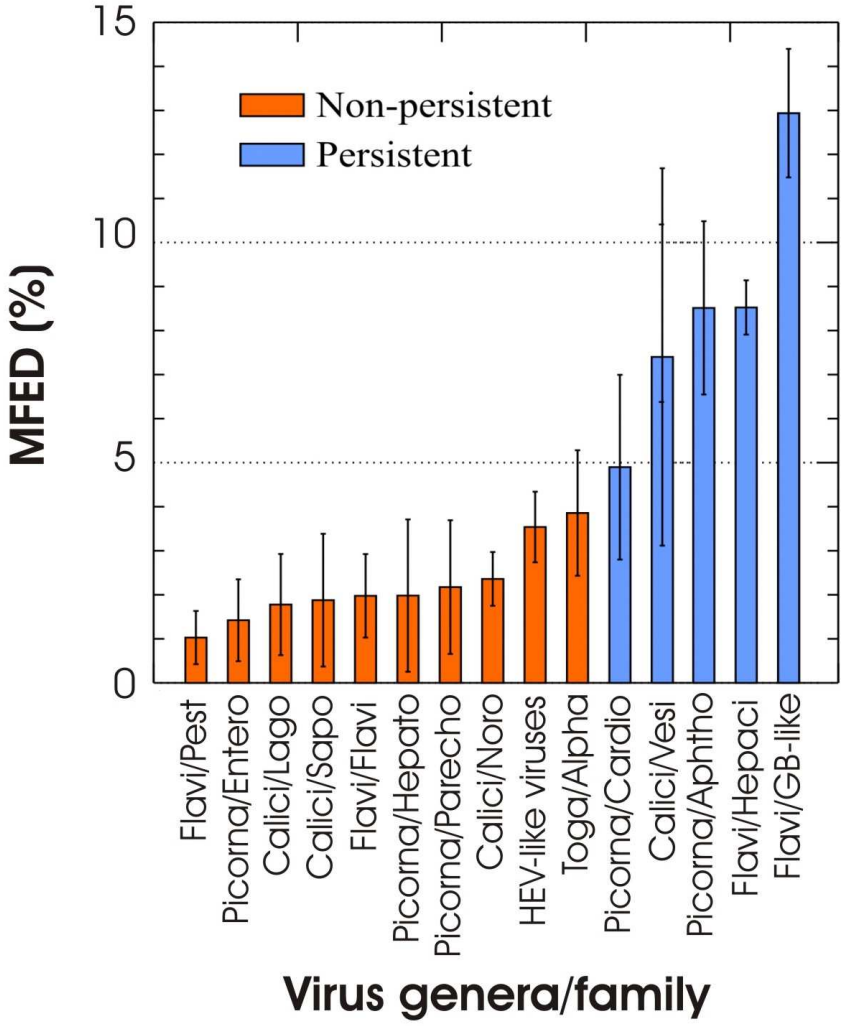


Figure 1.11 Correlation between secondary structure and persistence. Non-persistent viruses (orange) have lower % MFEDs than persistent viruses (blue).

The mechanism by which GORS may enable viruses to persist is not yet clear, although the avoidance of the dsRNA recognition pathways of the innate

immune system by, in some way, mimicking cellular RNA structures. It seems also, that GORS is an evolutionarily conserved phenomenon, as experimental drift of just 2 % sequence change reduced the MFED, and 10 % drift reduced the MFED of GORS viruses into that of non-GORS viruses, so maintaining a high degree of secondary structure throughout the genome must be advantageous for those viruses that contain it (Simmonds *et al*., 2004).

Recent work has investigated the physical characteristics of GORS, which had previously been only a bioinformatically predicted phenomenon. Probe hybridization was carried out on the genomes of predicted GORS viruses: HCV, GBV-C and MNV; and non-GORS viruses: enterovirus C, tick-borne encephalitis virus, rubella virus and bunyamwera virus. The GORS genomes were largely inaccessible to the probes, whereas the non-GORS viruses were able to hybridize with the probes throughout the majority of the genome. Atomic force microscopy of viral RNA transcripts of GORS and non-GORS viruses revealed that GORS RNA (HCV and GBV-C) appeared as tightly compacted spheroids whereas the non-GORS viruses (poliovirus and rubella virus) were pleomorphic with extensively single-stranded RNA (Davis *et al*., 2008).

1.7 Project aims

The aim of this project was conduct the first study into the role of GORS in the persistence of certain positive-sense RNA viruses. There were two lines of investigation:

i) Study of GORS viruses - as many of the predicted-GORS viruses are difficult to work, e.g. hepatitis C virus (HCV) and foot-and-mouth disease virus (FMDV) require category 3 containment facilities, the viruses chosen for study in this project were ERAV and MNV. The aim of this study was to:

- determine prevalence of ERAV in horses admitted to the Equine Hospital, R(D)SVS
- determine prevalence of MNV in cohorts of laboratory and wild mice
- complete genome sequencing of novel viruses identified for phylogenetic and bioinformatics analysis
- investigate effect of multiple passage in cell culture on the degree of secondary structure present in viral genomes

ii) Study of the effect of GORS on RNA stability and the interaction of viral RNA with cellular innate immunity – these studies addressed the observation that GORS viruses are able to establish persistent infection and tested the following hypotheses:

- RNA transcripts of GORS virus genomes are more stable in the cell cytoplasm than RNA transcripts of non-GORS virus genomes.
- RNA transcripts of GORS virus genomes induce IFN-β less strongly than RNA transcripts of non-GORS virus genomes.

Chapter 2 Materials and Methods

2.1 Cell culture

2.2 PCR screening of ERAV and MNV

2.3 Sequencing of ERAV and MNV genomes

2.4 Phylogenetic analysis

2.5 Prediction of free folding energy and RNA structure prediction

2.6 Virus growth and titration

2.7 Creation of RNA transcripts

2.8 RNA stability study

2.9 4 kilobase transcripts

2.10 Interferon induction study

Chapter 2 Material and Methods

2.1.1 Cell Culture

Cell lines used: CRE3T3 (murine fibroblast), NIH3T3 (murine fibroblast), RD (human rhabdomyosarcoma) cells, Vero 136 (African green monkey kidney epithelial cells) MDCK (Madin-Darby Canine Kidney), RAW 264.7 (murine leukaemic monocyte macrophage), A549n (human alveolar basal carcinoma) and A549NPro cells (expressing Bovine Viral Diarrhoea Virus NPro protein). All cell lines grow as adherent monolayers.

All cell lines were maintained in sterile plastic flasks (80 cm^2 surface area (T80)) (Nunc) at 37°C in a humidified 5 % CO_2 atmosphere. All cell lines were grown in Dulbecco's modified Eagles Medium (DMEM) (Invitrogen) supplemented with 10 % (v / v) foetal calf serum (FCS) (Invitrogen, UK), L-glutamine (200mM Invitrogen), penicillin (100 U / ml, Invitrogen) and streptomycin (100 µg / ml, Invitrogen).

2.1.2 Cell Counting

Cell lines were maintained by splitting the cell population twice a week, at intervals of 3 or 4 days. At a confluency of approximately 90 % the cell monolayers were washed briefly with phosphate buffered saline (PBS). To remove the monolayers from the flasks the majority of cell lines were incubated with Tryspin-ethylene diamine tetraacetic acid (EDTA) (0.05 % Trypsin, 0.53 mM EDTA, Invitrogen) in variable conditions for each cell line until the cells had detached from the surface of the flask:

RAW 264.7	Cells weakly adherent, trypsin not required as cells could be detached by repeated pipetting in culture medium alone
CRE3T3 / NIH3T3	Cells incubated with trypsin at room temperature for ~1 minute
RD	Cells incubated with trypsin at room temperature for ~2 minutes
Vero 136	Cells incubated with trypsin at room temperature for ~2 minutes

A549n / Npro	Cells incubated with trypsin at 37°C for ~2 minutes
MDCK	Cells incubated with trypsin at 37°C for up to 20 minutes

Table 2.1 List of cell lines used in this project

The trypsin was inactivated with an equal volume or excess of 10 % FCS tissue culture medium. The cells were collected by centrifugation at 478 x gravity (g) for 5 minutes. The cell pellet was resuspended in 10 ml of tissue culture medium. Ten microlitres of this suspension were added to a haemocytometer and the cells were counted; when considered necessary 10 µl of cell suspension were added to an equal volume of 0.4 % trypan blue (Sigma) in order to assess the proportion of live to dead cells. An appropriate number of cells were added to a fresh flask, e.g. for most cell lines 2 x 10^6 cells were added to a T80 flask, although RAW 264/7 cells are smaller than the other cells so ~4 x 10^6 were added.

2.1.3 Freezing and thawing cells

Cells were periodically frozen and thawed as necessary. Before freezing cells were grown to approximately ~80 % confluency when they were still in the log phase of growth. The monolayers were washed with PBS, trypsinised where necessary, centrifuged, resuspended and counted as previously described. Freezing mix, consisting of 90 % FCS and 10 % dimethyl sulphoxide (DMSO), was made to allow 1 ml per vial at 2-5 x 10^6 cells per cryovial (Nunc). Cells were then frozen at -80°C in a freezing container (Nalgene) containing 100 % isopropyl alcohol to ensure a cooling rate of 1°C / minute. The cryovials were transferred the following day to storage in liquid nitrogen at a temperature of approximately -196°C.

Before thawing cells, cell culture medium was warmed in a 37°C water bath and 20 ml were pipetted into a universal tube for each vial of cells to be thawed. Cryovials were removed from liquid nitrogen and placed on dry ice to prevent gradual thawing. The vials were then thawed rapidly in the 37°C water bath. Several drops of medium were added to each vial to equilibrate the osmotic pressure slowly. The cells were then added to the 20 ml of medium in the universal and centrifuged at 359 x g for 5 minutes at room temperature. The

supernatant was gently poured off, the cells resuspended in 7 ml of medium and added to a 25 cm^2 flask (T25). The following day the medium was changed in order to ensure that there was no DMSO remaining. The cells were moved up to the next sized flask as soon as they were growing well and had become confluent in the T25.

2.2 PCR screening of ERAV and MNV

2.2.1 Collection and processing of equine samples

Samples of ~2 ml of equine blood were taken by veterinary surgeons from horses at the Large Animal Hospital at Easter Bush Veterinary Centre as a diagnostic veterinary procedure. The blood was allowed to stand for approximately 30 minutes before being centrifuged at 3000 rpm for 5 minutes. The serum was decanted into fresh tubes and stored at -20°C at the clinical laboratory of the Veterinary Pathology Unit at the Easter Bush Veterinary Centre.

Equine faecal samples were collected from the horse boxes housing equine in-patients at the Large Animal Hospital at Easter Bush Veterinary Centre. As the boxes were cleaned every day the faecal samples would have been less than 1 day old. The faecal samples, weighing 1-3 g, were soaked in PBS at 3 ml PBS per 1 g faecal material for 6-8 hours with brief vortexing every 30 minutes in universal tubes. The faecal suspensions were centrifuged at 4000 x g for 20 minutes. One millilitre of the supernatant was centrifuged additionally at 14926 x g for 10 minutes. The supernatant was stored at -20°C for subsequent RNA extraction.

Isolates of ERAV and ERAV passaged from clinical samples were also kindly provided by Javier Castillo Olivares of the Animal Health Trust (Newmarket, UK).

2.2.2 Collection and processing of murine faecal samples

Mouse faecal samples were collected from thirty-nine cages in the animal unit at the University of Edinburgh by Mr Billy Smith. Each tube contained faecal pellets from a group of in contact animals. Up to 0.1 g of faecal material was suspended in 1 ml of PBS and allowed to soak for 2-3 hours. The faeces were then mechanically disrupted with a pipette tip and vortexed for 2 minutes. The faecal suspensions were clarified by centrifugation at 4000 x g for 20 minutes. The supernatants were passed through a 0.2 µm filter before being stored at -20°C. Murine faeces were also collected with kind permission from Morningside Pets, Edinburgh and processed as described above. Wild house mice (*Mus musculus*) were kindly collected in standard house traps by various people at the University of Edinburgh. The mice were killed under schedule 1 of the Animals (Scientific Procedures) Act by inducing deep anaesthesia by exposure to gaseous halothane (Concord Pharmaceuticals Ltd) followed by cervical dislocation. The mice were dissected and the faecal pellets removed directly from the rectum. Faeces were then processed as described above. Three species of wild rodent, wood mouse (*Apodemus silvaticus*), field vole (*Microtus agrestis*) and bank vole (*Myodes galveolus*) had been trapped, euthanased and stored at -70°C by Anna Meredith, University of Edinburgh, who kindly allowed access to these animals for the collection of tissue and faecal samples. Small samples of spleen and mid-jejunum were placed in 0.5 ml of RNA Later (Qiagen) and stored at -20°C. Faecal samples were processed as described above.

2.2.3 Extraction of viral RNA

Extraction of viral RNA from faecal suspensions was carried out using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's recommended protocol. The lyophilised carrier RNA was resuspended in the buffer AVE to a concentration of 1 µg / µl before adding 5.6 µl carrier RNA solution to 5.6 ml of the lysis buffer AVL per sample. Carrier RNA is added to enhance the binding of viral nucleic acids to the Qiamp Mini membrane and to protect viral RNAs from degradation from any remaining RNases. One hundred and forty microlitres of faecal suspension was added to 560 µl buffer AVL containing carrier RNA, mixed by vortexing for 15 seconds (s) and incubated at

room temperature for 10 minutes. Five hundred and sixty microlitres of 70 % ethanol were added to each sample and mixed by vortexing for 15 s. Six hundred and thirty microlitres of the solution was added to the QIAamp Mini column and centrifuged at 6000 x g for 1 minute, the filtrate was discarded and the remainder of the solution added to the column and the spin repeated. Buffers AW1 and AW2 were diluted with 100 % ethanol as directed. Five hundred microlitres of AW1 were added to the column and centrifuged at 6000 x g for 1 minute; then 500 µl of AW2 were added to the column and centrifuged at 20000 x g for 3 minutes. The columns were placed into an RNase free tube, 60 µl of buffer AVE added, incubated at room temperature for 1 minute before the RNA was eluted by centrifugation at 6000 x g for 1 minute

2.2.4 Reverse transcription

Reverse transcription of viral RNA into cDNA was carried out using the Reverse Transcription System (Promega). 5 µl of RNA were added to RNase free tubes and heated to 70°C for 10 minutes and placed on ice. The following reaction mix was then prepared:

Nuclease-Free Water	-	4.8 µl
$MgCl_2$, 25 mM	-	4 µl
Reverse Transcription 10x Buffer	-	2 µl
dNTP Mixture, 10 mM	-	2 µl
Random Primers, 0.5 µg / ml	-	1 µl
Recombinant RNasin Ribonuclease inhibitor	-	0.5 µl
AMV Reverse Transcriptase, 22 U / µl	-	15 U / 0.7 µl

15 microlitres of mix were added to the 5 µl RNA to make a 20 µl reaction volume. The samples were incubated at room temperature for 10 minutes then at 42°C for 50 minutes. The AMV reverse transcriptase was deactivated by heating the samples at 95°C for 5 minutes then incubated on ice for 5 minutes. The cDNAs were then stored at -20°C.

2.2.5 Design primers for screening of ERAV and MNV

Primers were designed for screening from consensus sequences made from published sequences of ERAV and MNV. ERAV primers were designed in the capsid region as well as the 5' UTR. Screening primers were also made for screening wild rodent samples from a consensus sequence consisting of published MNV and human norovirus sequences. Primers were designed as a nested polymerase chain reaction (PCR) to increase sensitivity and specificity; the broad norovirus PCR was hemi-nested as the same antisense primer was used in both primary and secondary reactions.

Primer	Orientation	Sequence	Product length
ERAV capsid OS	Sense	CCNGGTGACAARCTSAARAAGATGGGYAG	391
ERAV capsid OAS	Antisense	ACYGACATGGTCACRGGCACWGTTTG	
ERAV capsid IS	Sense	CACCACCTGGTCAAGAAYGGMTGGGA	145
ERAV capsid IAS	Antisense	GCTGGTTCYTCMARCTCAGACCAYTT	
ERAV screen OS	Sense	GGCAAGCTGGTTACAGCCCTGA	188
ERAV screen OAS	Antisense	CCATCATSCGAACRGCRCCTGC	
ERAV screen IS	Sense	AGCAGCAGCAGRAACGCGGGAG	98
ERAV screen IAS	Antisense	CCTGCYAGYAGAGTCTGCTCGC	
MNV capsid OS	Sense	TCAGCAGTCTTTGTGAATGAGG	482
MNV capsid OAS	Antisense	TCAAGAAGAGGGAGTTGAATGG	
MNV capsid IS	Sense	ACCCCAGGTGAAATACTGTTTG	177
MNV capsid IAS	Antisense	TGGGAAAATAGGGTGGTACAAG	

Noro 1729 OS	Sense	SCCCMSTCACKCTVAAVTGTG	306
Noro 2035 AS	Antisense	CCYTTSCCRTRNGGGGTRTT	
Noro 1745 IS	Sense	YTGTGAYAGRATTGARAAYAAR	290
Noro 2035 AS	Antisense	CCYTTSCCRTRNGGGGTRTT	

Table 2.2 List of screening primers for ERAV, MNV and norovirus

The primers contain the following degenerate bases:

R – A or G	**H** - A or T or C
Y – C or T	**B** – G or T or C
M – A or C	**D** – G or A or T
K – G or T	**V** – G or A or C
S – G or C	**N** – A or C or G or T
W - A or T	

Table 2.3 List of degenerate bases.

2.2.6 Polymerase chain reaction (PCR)

Polymerase chain reaction is a technique used to amplify specific DNA sequences from template DNA. Sense and antisense primers are designed to anneal specifically to their corresponding DNA strands. During the reaction the DNA double helix is denatured before the primers anneal. The primers are extended by the action of the DNA polymerase adding complementary nucleotides during the extension phase. The cycle is repeated multiple times creating a predominance of the specific product.

The following reaction mix was prepared using a reaction buffer containing 50 mM Tris-HCl (pH 8.5), 50 mM NaCl and 7.5 mM $MgCl_2$; 3 mM dNTP (Promega); primers at 10 OD (optical density at 260 nm) (Sigma) and GoTaq DNA polymerase (Promega):

Nuclease free water	-	13.5 µl / 14.5 µl
5x Colourless GoTaq Reaction Buffer	-	4 µl
dNTPs, 3 mM	-	0.2 µl
Sense primer, 10 OD	-	0.1 µl
Antisense primer, 10 OD	-	0.1 µl

GoTaq DNA Polymerase, 5 U / µl	-	0.1 µl
Template / primary PCR product	-	2 µl / 1 µl

The primary PCR reaction consisted of 18 µl of mix plus 2 µl of template cDNA; the secondary PCR reaction consisted of a 19 µl reaction plus 1 µl of primary PCR product.

The following PCR reaction program was run:

No. of cycles	**Temperature (°C)**	**Time**
1	95	2 min
30	95 50 72	30 s 30 s 90 s
1	72	5 min

Table 2.4 Standard PCR conditions

2.2.7 Agarose gel electrophoresis

A 2 % agarose gel was made by dissolving 6 g of SeaKem LE agarose (Cambrex) in 300 ml of 1x TAE buffer (50x TAE buffer made by dissolving 242g Tris base (VWR), 57.1 ml glacial acetic acid (Sigma) and 100 ml 0.5 M EDTA (Fisher Scientific) in 1 l of water. The agarose was dissolved by heating in a microwave oven on full power for 5 minutes and allowed to cool whilst being mixed by a magnetic stirrer. Thirty microlitres of ethidium bromide, at 10 mg / ml (Fluka) were added to the gel prior to pouring into a gel tray. After the gel had set it was stored at 4°C. Nine microlitres of secondary PCR product were mixed with 1 µl of 10x loading dye (containing 0.1 M EDTA (Fisher Scientific); 0.1 % bromophenol blue (Fisher Scientific); 0.1 % xylene cyanol (Fisher Scientific); 1.75 M sucrose (Fisher Scientific)) and gels run at 150 V for 30-40 minutes. The Low Range Plus DNA Ladder (Fisher Scientific) or 100 bp Ladder (Promega) were run alongside samples. Bands were visualised using an ultra-violet transilluminator.

2.2.8 PCR of two regions of MNV for phylogenetic analysis

After screening murine samples for MNV, any PCR positive samples underwent further PCRs with the purpose of determining the sequence of the PCR products for phylogenetic analysis. Two regions were selected, one in the non-structural region, the other in the structural region and primers designed for a nested PCR for each to create products of 790 and 782 bases in length.

Region	Sense	Start	Sequence	Product length
L1	OS	892	TCGTGTTGAGCTGTGAYTGGAC	917
NS3	OAS	1809	GRTTGGTGGTGATGATGATGAC	
	IS	991	ACTGGATGATCTCYATCTTTGG	790
	IAS	1781	GTCAAACATCTTCCCCTTRTTC	
L2	OS	5973	GACMTTCACCMTGATTGAGCAG	993
VP1-ORF3	OAS	6966	TGTTGATGGCRTTCTCCTGGGC	
	IS	6112	GTSACCACYTTTGAGATGATYC	782
	IAS	6894	AAGAGTTGGYTTGGAGCTGCTG	

Table 2.5 Primers used for PCR of two MNV sequences for use in phylogenetic analysis

2.3 Sequencing of ERAV and MNV genomes

2.3.1 Design of primers for complete genome sequencing

Primers were also designed for sequencing the length of the genomes of ERAV and MNV. Primers were designed so that each PCR product would be approximately 800 bases long. The sequencing reaction gives reliable sequences of approximately 500 bases starting from both the sense and antisense primers, so a product length of 800 bases ensures that there is no gap. The primer set were also designed to overlap by approximately 100 bases to ensure agreement between the different sequencing reactions.

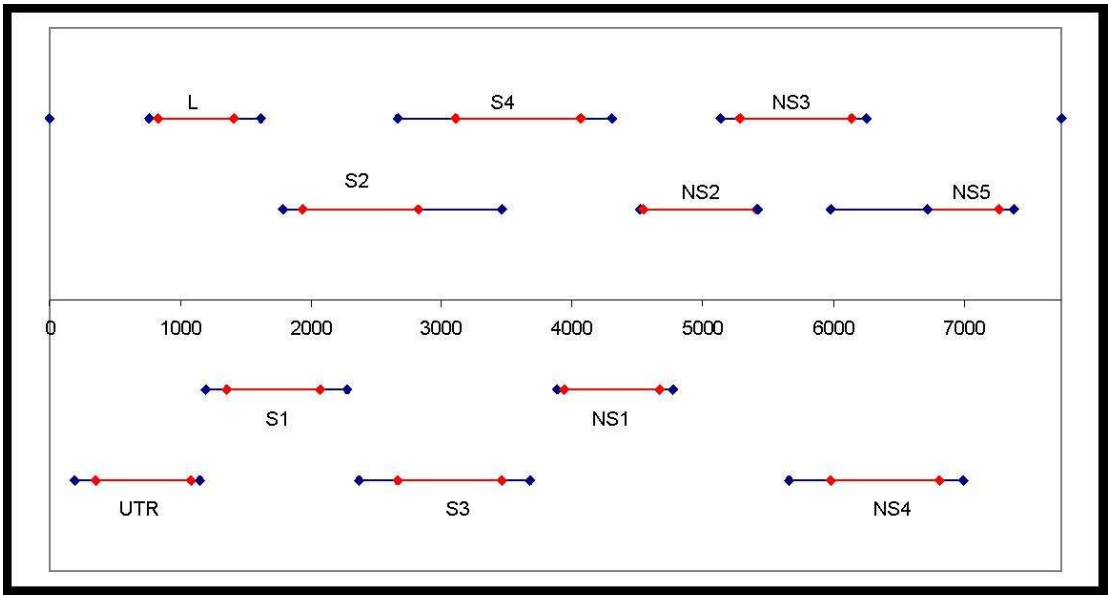


Figure 2.1. Position of primers on ERAV genome. Red and blue lines show sets of nested primers; blue dots and lines represent outer primers in each set, the red lines represent inner primer sets. The products of inner primer PCRs were sequenced.

Name	Sense	Start	Sequence	Product length
UTR	OS	189	CAGAGCTTCCCGGCTTTAAGGGTTAC	378
	OAS	567	GTGTCGCTTYGTRAGGTTCACTCCGC	
	IS	294	CTTKTCTCYYAAACAATGTTGGCGCGC	229
	IAS	523	ACCATCCAGAGCAATGGCACCGGGAA	
UTR2	OS	189	CAGAGCTTCCCGGCTTTAAGGG	963
	OAS	1152	TGGTCACTCACCCAATCAATTG	
	IS	348	CCTGTCATTGACTGGTCGAAGG	734
	IAS	1082	GAATYCCAAGAATCTGTGTCAG	
L	OS	762	GGCAAGCTGGTTACAGCCCTGA	854
	OAS	1616	CTGCATCAATTGARTTYTGGTA	
	IS	835	AGCAGCAGCAGRAACGCGGGAG	619
	IAS	1454	TGCCCARTGACTCAAAGTCATA	
S1	OS	1200	TGTACAAAYTRTGGCAACATGG	1082
	OAS	2282	CAATRTAGGGCATVACCAAATG	
	IS	1357	RGGTTGGATYTGTGTYGATGAC	721
	IAS	2078	CCACATCCCAKCCRTTCTTSAC	
S2	OS	1787	ATTGARACAACNGTGGTTGGRG	1672
	OAS	3459	GTCTTRGTTGGTGCYCCAACMG	
	IS	1933	CACHTTYAARGTDGGYGAGTGG	888
	IAS	2821	GTGRGGRGGCACAAAMGCMACC	
S3	OS	2369	ACWGTRCCYGTGACCATGTCRG	1315
	OAS	3684	GCACCDGGMAGCCAATTGTARG	
	IS	2663	ATGGATGTRTCBCTCAGYGCDG	796
	IAS	3459	GTCTTRGTTGGTGCYCCAACMG	
S4	OS	2663	ATGGATGTRTCBCTCAGYGCDG	1644
	OAS	4307	CRACCACTCCTTCAGGSGTTTC	
	IS	3104	CCYGAYAAGCAGGTYACHAAYG	966
	IAS	4070	CYGYCACAGCYAGTGTTGCAAG	

NS1	OS	3882	CTGGAGATGTTGAGAGCAACCC	884
	OAS	4766	CATCAAAATATGTRGGGTCWGG	
	IS	3944	GCMTTGTCAACRTCRCTRGGTG	726
	IAS	4670	AAGATTTKCCYTGYCCTGATGC	
NS2	OS	4517	CGCCAGTGGTTYAARGACATGC	907
	OAS	5424	CTYCTRGTCATRTATGACTCCCA	
	IS	4541	CAGCGTGCTCTYGCYGTGAARG	864
	IAS	5405	CCCAYTTRAGATACTCTTCATG	
NS3	OS	5139	ATGGRCAGGCTGTTAAATTGGC	1112
	OAS	6251	CAAGRCGAMYAACGTTGCCCTG	
	IS	5282	AARTGYWCAACTGATGAGGARC	853
	IAS	6135	GCAGGTGAGCCWGCMACAATTG	
NS4	OS	5656	GAARAATGTTCAGCCAATTGAG	1339
	OAS	6995	CTCTCATTGCATCAGCTGCATG	
	IS	5978	TCHCCACACCARGCACGYACTG	837
	IAS	6815	GYTGCCTACCAACYACCACATG	
NS5	OS	5978	TCHCCACACCARGCACGYACTG	1402
	OAS	7380	AARGTSACCTCYGAAAGCGTTC	
	IS	6722	AAAGATGAGATYCGCCCACTTG	543
	IAS	7265	CAGAACGAATGAGGCARTCRTC	

Table 2.6 Primer sets used in almost complete genome sequencing of ERAV

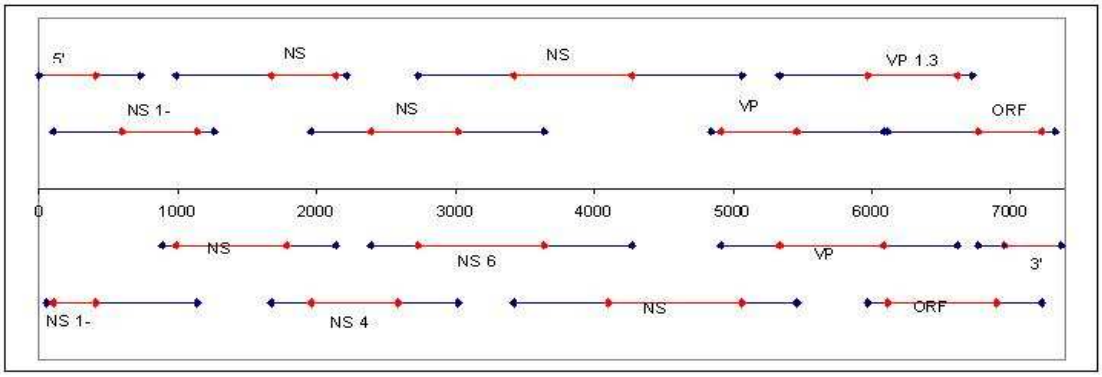


Figure 2.2. Position of primers on MNV genome. Red and blue lines show sets of nested primers; blue dots and lines represent outer primers in each set, the red lines represent inner primer sets. The products of inner primer PCRs were sequenced.

Name	Sense	Start	Sequence	Product length
5’	OS	1	GTGAAATGAGGATGGCAACGCC	726
	OAS	727	TAGAGATCTTCGCCCTCTTCAG	
	OS	1	GTGAAATGAGGATGGCAACGCC	406
	IAS	407	CTCYYTTGCATCCATRGCGTCC	
NS1-2.1	OS	58	GCAAAAACAAAAAGGCTTCATC	1081
	OAS	1139	CGAGTTGGTGGAGGCGAGCTTG	
	IS	100	GAGCACCTAGCCTTCTCTCTTC	307
	IAS	407	CTCYYTTGCATCCATRGCGTCC	
NS1-2.2	OS	100	GAGCACCTAGCCTTCTCTCTTC	1164
	OAS	1264	ATGACAGCCTGCTCCATGTTGG	
	IS	599	GTTGAGGAAGTGYGTTGGCATG	540
	IAS	1139	CGAGTTGGTGGAGGCGAGCTTG	
NS3.1	OS	892	TCGTGTTGAGCTGTGAYTGGAC	1244
	OAS	2136	ARTCATAGACCTTGTTYTGGAG	
	IS	991	ACTGGATGATCTCYATCTTTGG	790
	IAS	1781	GTCAAACATCTTCCCCTTRTTC	

NS3.2	OS	991	ACTGGATGATCTCYATCTTTGG	1221
	OAS	2212	ATCGCTGCCATCTTGTACCARG	
	IS	1674	GGCATGGACAAYGTGGTGAAGG	462
	IAS	2136	ARTCATAGACCTTGTTYTGGAG	
NS4	OS	1674	GGCATGGACAAYGTGGTGAAGG	1344
	OAS	3018	CAACACGGGACCAGATGGARAC	
	IS	1961	CTACAGCCACATCAAYTTCATC	626
	IAS	2587	TCCTCAAAGAGRTCAAAGGCAC	
NS5	OS	1961	CTACAGCCACATCAAYTTCATC	1677
	OAS	3638	CCAGAACATGGTCTTGGTGATC	
	IS	2391	TGTGTCAGRAGGATYAAGGAGG	627
	IAS	3018	CAACACGGGACCAGATGGARAC	

NS6	OS	2391	TGTGTCAGRAGGATYAAGGAGG	1877
	OAS	4268	ATCCATGTGGTAYCTGAARTTG	
	IS	2732	CAAGTACTCCATTGATGAYTAC	906
	IAS	3638	CCAGAACATGGTCTTGGTGATC	
NS7.1	OS	2732	CAAGTACTCCATTGATGAYTAC	2335
	OAS	5067	CATCCTCATTCACAAAGACTGC	
	IS	3423	TAYAAGAARGGAAACACCTGGG	845
	IAS	4268	ATCCATGTGGTAYCTGAARTTG	
NS7.2	OS	3423	TAYAAGAARGGAAACACCTGGG	2032
	OAS	5455	TGGGAAAATAGGGTGGTACAAC	
	IS	4099	TYTGGGGCTCTGAYCTTGGCAC	968
	IAS	5067	CATCCTCATTCACAAAGACTGC	
VP1.1	OS	4840	CAGGACAYGCTCAGAGACCCTC	1245
	OAS	6085	TCTCRGTCTGSACCTCGATCTC	
	IS	4919	TGTGGCTTCCCGGGTCTCCAAG	536
	IAS	5455	TGGGAAAATAGGGTGGTACAAC	

VP1.2	OS	4919	TGTGGCTTCCCGGGTCTCCAAG	1697
	OAS	6616	AGCTGACGACCTCAAAGAAGCC	
	IS	5334	TGCCCACCTCTCAGCCATGTAC	751
	IAS	6085	TCTCRGTCTGSACCTCGATCTC	
VP1.3	OS	5334	TGCCCACCTCTCAGCCATGTAC	1383
	OAS	6717	CACCTCCAATCGCTCCRAARAG	
	IS	5973	GACMTTCACCMTGATTGAGCAG	643
	IAS	6616	AGCTGACGACCTCAAAGAAGCC	
ORF3.1	OS	5973	GACMTTCACCMTGATTGAGCAG	1251
	OAS	7224	GRGACCAGGAACCTCCACGAAG	
	IS	6112	GTSACCACYTTTGAGATGATYC	782
	IAS	6894	AAGAGTTGGYTTGGAGCTGCTG	

ORF3.2	OS	6112	GTSACCACYTTTGAGATGATYC	1209
	OAS	7321	TCTCTTCCAACCTATGCCCTGC	
	IS 1	6765	TCARAACCTTCAGGCAAATAAC	459
	IAS	7224	GRGACCAGGAACCTCCACGAAG	
3’	OS	6765	TCARAACCTTCAGGCAAATAAC	601
	OAS	7366	GCAGTAAGCAGAAATCATTTTC	
	IS	6957	CCAGGAGAAYGCCATCAACATC	409
	OAS	7366	GCAGTAAGCAGAAATCATTTTC	

Table 2.7 Primer sets used in almost complete genome sequencing of MNV

2.3.2 Sequencing reaction

For sequencing reactions, the following reaction mix was assembled:

Nuclease free water	-	17 µl
Sense or antisense primer, 10 OD	-	1 µl
Big Dye (Applied Biosystems)	-	1 µl
PCR product	-	1 µl

The following reaction was carried out:

No. of cycles	**Temperature (°C)**	**Time**
30	96	30 s
	50	20 s
	60	4 min

Sequencing reaction conditions

The products of the sequencing reaction were sent to The Gene Pool, The University of Edinburgh School of Biological Sciences. Sequence data were returned in the form of a chromatogram, which could be visualized using Chromas Lite (www.technelysium.com.au), and an accompanying file containing a sequence readout. The sequence files were converted into a FASTA format and imported into Simmonics v1.73 for analysis and editing; sequence files were checked against the chromatogram for accuracy. Published sequences of ERAV and MNV were also imported in FASTA format from NCBI Entrez Nucleotide (www.ncbi.nlm.nih.gov/sites/entrez) for use in helping to construct received sequences and generating phylogenetic trees.

2.3.3 Genome sequencing of a novel norovirus

During screening of wild rodent faecal samples with the degenerate norovirus primers a positive result was seen in one sample from a wood mouse (*Apodemus silvaticus*). A PCR reaction was carried out with each of the MNV primer sets along the full length of the genome. Three primer sets gave a positive result, these products were sequenced which gave sequence data at positions 991 – 2587 and 5476 – 5954. In order to complete the rest of the genome sequence, specific primers were designed from the newly acquired sequences to enable approximately 500 more bases to be sequenced. New primers were designed and a combination of specific primers and MNV primers were used in the PCRs and sequencing reactions until the gap in the between the two originally sequenced parts of the genome had been closed. To sequence the regions between the original sequences and the 5' and 3' ends of the genome specific primers were designed and used with a range of MNV primers in nested PCRs. Sequences obtained from these reactions could likewise be used to design further specific primers to sequence the length of the entire genome. The primers designed specific to the novel norovirus are named "Aponoro" followed by the position on the genome and sense:

Aponoro 433 AS	434-413	CTCAAGAATGGATCCAACCTTG
Aponoro 1110 S	1110-1131	TTCTGAGACTCTTGGGAGGAAG
Aponoro 1342 S	1342-1363	TTCCTTAAAGCCCTTGATGATG
Aponoro 1566 AS	1566-1545	CAGATTCTGGCAAAACCAAGTC
Aponoro 1679 AS	1679-1658	TCATCCCATACAACAACTCTGG
Aponoro 1868 S	1868-1889	TGACTTTTTGGTCTACGCTGAG
Aponoro 2030 AS	2030-2009	GTGTTTCCTTGTCGGTCAAAAC
Aponoro 2198 S	2198-2219	TCCCTTGGTATAAGATGGCAAC
Aponoro 2452 S	2452-2473	ACTGATGTGGTGTCAACTGTCC
Aponoro 2802 S	2802-2823	TTTGAAGAAATGTTTGGTGACG
Aponoro 3263 S	3263-3284	CTCTGGAGAGATGCTTGCTTTG
Aponoro 4754 AS	4754-4733	ACCGAACTGGTCACCAACTATC
Aponoro 5295 AS	5295-5274	AATCTCACCTGGGGTATTTCTG
Aponoro 5565 S	5565-5586	GTGGCATGCTACTCAAGATCAG
Aponoro 5648 S	5648-5669	ATGAGTCATTTGTGGTGTCTGG

Aponoro 5670 AS	5670-5649	ACCACAAATGACTCATCACCAG
Aponoro 5700 AS	5700-5721	ACAGCAGGCCTCGATAGTCGTC
Aponoro 7150 S	7150-7171	TGGTTGCTCGCTCTGTGGACAC

Table 2.8 Primers specific for *Apodemus* MNV.

The following primer sets were used in the PCR reactions followed by the sequencing reaction of positives and analysis as described. Multiple combinations of Aponoro and MNV primers were used to maximise the chance of a positive PCR result which would lead to a product that could be sequenced. Table x shows the primer sets used in the nested PCRs used for the generation of the complete genome sequence.

Outer sense	**Inner sense**	**Inner antisense**	**Outer antisense**
MNV 1	**MNV 1**	**Apo 433**	Apo 1566
MNV 58	**MNV 110** **MNV 599** **MNV 892** **MNV 991**	**Apo 1566**	Apo 1679
MNV 991	**MNV 1674**	**MNV 2136**	MNV 2212
Apo 1342	**Apo 1868**	**Apo 2288**	Apo 2288
MNV 1674	**MNV 1961**	**MNV 2587**	MNV 3018
Apo 2198	**Apo 2802**	**Apo 4754**	Apo 5295
Apo 2198	**Apo 2452**	**Apo 5670**	Apo 5700
	Apo 2802	**Apo 5275** **MNV 3018**	
MNV 4840	**MNV 5334**	**MNV 6085**	MNV 6616
Apo 5565	**Apo 5648**	**MNV 6085** **MNV 6616** **MNV 6717** **MNV 6894** **MNV 6955** **MNV 7224**	MNV 7224 MNV 7321
Apo 5565	**Apo 5648**	**MNV 7356**	MNV 7356

Table 2.9 Primer sets fro complete genome sequencing of *Apodemus* MNV, a mixture of specific *Apodemus* MNV and MNV

2.4 Phylogenetic analysis

Phylogenetic trees are a graphic representation of evolutionary relationships between genetic sequences within or between species. Phylogenetic trees were constructed from novel and previously published MNV sequences by neighbour joining using the Jukes-Cantor model with pair-wise deletion for missing nucleotides in Mega 4 (Mega 4.0.1, Tamura, Dudley, Nei, and Kumar 2007). Pairwise distances were also calculated by Mega 4.

2.5 Prediction of folding free energy and RNA structure prediction

RNA secondary structure prediction along the length of the genome was carried out on 4 complete MNV genomes from laboratory mice, one full length genome from the pet shop mouse and the full length genome of the wood mouse MNV. The degree of secondary structure along the genome was also predicted for MNV1-4 taken from GenBank. Folding free energy calculations were made using UNAFold (http://dinamelt.bioinfo.rpi.edu/refs.php; Markham, N. R. & Zuker, M. (2008)). Minimum free energy differences (MFED) were calculated by comparing the MFE of the native sequence to 50 randomised control sequences retaining the same nucleotide composition. This determines the degree of secondary structure dependent on sequence order. The sequence randomisation algorithm used was NDR which preserves dinucleotide frequencies. The genome was divided in fragments of 300 bases, starting at position 58, spaced at intervals of 9 bases. This created 776 overlapping fragments for which the MFEDs were calculated in both the sense and antisense directions. Sequence randomisations were carried out using internal functions of the SIMMONIC editor package v 1.7.

2.6 Virus growth and titration

Virus	Cell type grown in
Murine Norovirus (MNV)	RAW cells
Equine Rhinitis A Virus (ERAV)	Vero136 cells
Canine Calicivirus (CaCV)	MDCK cells
Semliki Forest Virus A7 (74) (SFV)	CRE3T3 or NIH3T3 cells
Echovirus 11 (E11)	RD cells
Theiler's Murine Encephalomyelitis Virus (TMEV)	CRE3T3 or NIH3T3 cells

Table 2.10 Viruses used in this project and cell lines grown in

2.6.1 Infection of cells with virus and creation of virus stocks

To create a stock of each virus it was necessary to grow them up in cell culture. Viruses were generally grown up in a T175 flask or a T80 flask depending on the amount of material and the titre of the virus. Cells were grown to a confluency of approximately 70 %. Virus was added to the cells in a small volume of cell culture medium e.g. 10 ml in a T175 flask or 4 ml in a T80 flask. The cells were incubated with the virus in the incubator at 37°C for 1 hour. Cell culture medium was then added to the flasks up to 50 ml in a T175, up to 18 ml in a T80. The flasks were returned to the incubator and observed for signs of cytopathic effect (CPE). Cytopathic effect is the morphological change the cells undergo as a result of virus infection; it is often identified by the appearance of cell rounding, shrinkage, detachment from the flask and lysis. Dead cells may be seen floating within the culture medium. RAW cells have a round morphology but CPE is still easily identifiable by other morphological changes such as cell shrinkage. Virus was harvested from the cell culture medium at the time of marked CPE but before large numbers of cells died. The medium was aspirated from the flasks and transferred to universal tubes. The medium was clarified by centrifugation at 1328 x g for 20 minutes at 4°C. The supernatant containing the virus was divided into 2 ml aliquots which were stored at -80°C.

2.6.2 Titration of viruses

Viruses were titrated by using an endpoint method. A 96-well plate (Nunc) was prepared with 5 x 10^5 cells per well in a volume of 200 µl with all cell types except for RAW cells which were seeded at 1 x 10^6 cells per well. Plates were left overnight to allow cells to adhere to the wells. Eight serial 10-fold dilutions of each virus were made by adding 300 µl of each dilution to 2.7 ml of 2 % FCS cell culture medium. The medium was aspirated from the plate and 200 µl of each dilution was added to an entire row of the plate resulting in each of the eight dilutions being added to twelve wells. Plates were incubated at 37°C for 1-2 days and monitored for CPE and/or cell death. Tissue culture infective dose 50 ($TCID_{50}$) was calculated using the Reed-Muench method (Reed and Muench, 1932; as described in Fundamental Virology, Ed. D. Knipe and P. Howley; Chapter 1, A. Levine). The number of infected and uninfected wells were counted and recorded. A cumulative total of infected wells from the highest dilution to the lowest was created, as was a cumulative total of uninfected wells from the lowest dilution to the highest. A ratio was calculated to determine percentage of infected wells for each dilution. The dilution at which 50 % of the wells would be infected was then estimated by using the following equation:

$$\frac{(\%\ \textit{positive above}\ 50\%) - 50\%}{(\%\ \textit{positive above}\ 50\%) - (\%\ \textit{positive below}\ 50\%)} = \textit{proportionate distance}$$

The result of the equation gives the proportionate distance between the two dilutions that gave a percentage infection either side of 50 %. The distance between the dilutions is calculated by using the following equation:

(log dilution above 50%) + (proportionate distance x log dilution factor) = log $TCID_{50}$

The reciprocal of the $TCID_{50}$ is used to express the virus titre as $TCID_{50}$ / unit volume. $TCID_{50}$ / 200 µl was then converted into $TCID_{50}$ / ml.

2.6.3 Virus neutralisation assay

The equine serum samples were tested for the presence of antibody against ERAV by a virus neutralisation assay. Vero 136 cells were seeded into a 96-well plate at a density of 5 x 10^5 cells per well in a volume of 200 µl. The equine sera were diluted to 1:40, 1:160, 1:640 and 1:2560. Mixed with each serum dilution was ERAV at a titre of 10^2 $TCID_{50}$ / ml (the highest dilution that produce 100 % infected wells during virus titration). A positive control consisted of virus mixed with equine serum known to contain anti-ERAV antibodies (kindly provided by Tobias Tuthill); negative controls consisted of virus with no serum and uninfected cells. Each plate consisted of the four dilutions of each serum plus virus added to 2 wells. The results, i.e. wells infected or uninfected, were recorded on the table below:

	ERAV No serum		ERAV + sample serum		ERAV + sample serum		ERAV + sample serum		ERAV + sample serum		ERAV + sample serum	
1:160												
1:640												
1:2560												
1:10240												
1:160												
1:640												
1:2560												
1:10240												
	Uninfected cells		ERAV + sample serum		ERAV + sample serum		ERAV + sample serum		ERAV + sample serum		ERAV + Antibody (+ve cont)	

Figure 2.3 Table used in recording result of virus neutralisation assay.

2.6.4 MNV passage

In order to assess the effect of multiple passage of MNV3 on its genome a system was established to serially passage the virus at high and low multiplicities of infection. RAW cells were seeded in a 12-well plate (Nunc). Wells for the first passage were seeded at 5 x 10^5 cells per well for infection the following day; wells for the second passage were seeded at 3 x 10^5 cells per well for infection after 3 days. MNV virus stock was used; at high multiplicity of infection (moi) 400 µl of virus stock was added to the cells, at low moi 40 µl of virus were added to 360 µl of medium before being added to the cells, and mock cells had 400 µl of cell culture medium alone added. The virus was incubated with the cells for 1 hour then was removed and 1.5 ml of 2 % FCS medium was added. After 2 days the 400 µl of medium from the high moi infection and 40 µl of medium from the low moi infected cells were taken and the previously prepared wells infected in the same way. The remainder of the medium from the each passage was stored at -80°C. MNV3 was passaged thirty-three times. Passages 10, 20 and 33 were sequenced at the 2 parts of the genome used previously for phylogenetic analysis. The complete genome of passage 33 was sequenced.

2.7 Creation of RNA transcripts

2.7.1 Plasmids used in the generation of RNA transcripts

RNA transcripts of viral genomes from a panel of GORS and non-GORS viruses were made for use in RNA stability and interferon induction experiments. Viruses used were GB Virus-C (GBVC), hepatitis C virus JFH-1 GND (HCV), murine norovirus 1 (MNV1), pea enation mosaic virus (PEMV), bunyamwera virus (BV), poliovirus (PV), rubella virus (RV), Semliki Forest virus (SFV). RNA transcripts were made from the following full-length clones kindly provided by other collaborators.

pHGV (Iowan) (GBVC)	Jack Stapleton (University of Iowa)
pJFH-1 GND (HCV)	John Mclauchlan (MRC Virology Unit)
pSport MNV-1 20.3 (MNV1)	Ian Goodfellow (Imperial College, London)
pPEMV2 (PEMV2)	Eugene Ryabov, (University of Warwick)
pT7RiboBUN$_L$ (+) (BV)	Richard Elliott (University of St. Andrews)
pJM1 (Mah) (PV)	David Evans (University of Warwick)
pRobo502 (RV)	Teryl Frey (Georgia State University)
pSFV10 enh (SFV)	John Fazakerley (University of Edinburgh)
pBluescript_IISK_TMEVGD7 (TMEV)	Pablo de Felipe (St. Andrews)

Table 2.11 Infectious clones used to manufacture RNA transcripts; and source

2.7.2 Generation of RNA transcripts from plasmids

Full length and truncated RNA transcripts were made by Dr Matthew Davis. Briefly, each plasmid was linearised with the appropriate restriction enzyme (Davis *et al*. 2008). The DNA was then extracted by phenol-chloroform extraction and ethanol precipitated. As the purpose of generating the RNA transcripts for these studies was to study the effects of viral RNA within cells in the absence of viral replication, several of the DNA clones were digested with restriction enzymes to produce a truncated transcript incapable of replication.

Virus	Restriction enzyme	Truncation point	Nucleotides lost
GBV-C	MluI	8862/9395	533
HCV	EcoRV	9236/9678	442
PV	PvuII	7055/7440	385
TMEV	RsrII	6724/8101	1377
RV	BamHI	9174/9762	588

Table2.12 Restriction enzyme used to truncate plasmids

The other transcripts were made untruncated as MNV was replication incompetent, SFV consisted only of the non-structural genes and the BV transcript consisted only of the L segment. *In vitro* transcription was undertaken using the Megascript T7/SP6 kit (Ambion).

2.7.3 Enzymatic treatment of RNA transcripts

The RNA stability study was designed to measure the rate of degradation of RNA transcripts after electroporation into cells. As the rate of decay was measured by quantitative real-time PCR, it was essential to ensure that there was no plasmid DNA remaining. The transcripts were therefore incubated with 0.5 µl Turbo DNAse (Megascript Kit, Ambion) and 0.3 µlof the restriction enzyme HaeIII (New England Biolabs) per 10 µl of RNA.

2.8 RNA stability study

2.8.1 Electroporation of cells

Two large flasks (T175) of NIH3T3 cells were grown to near confluence. The cells were trypsinised and resuspended in 10 ml of cell culture medium; the cells were then counted. The cells were centrifuged at 544 x g for 5 minutes. The cell pellet was resuspended in 10 ml chilled PBS. The cells were washed in diethylpyrocarbonate (DEPC)-treated PBS and spun a further two times. After the last spin, the cell pellet was resuspended at a concentration of 2 x 10^7 cells / ml in 400 µl per electroporation. Then 400 µl cells were transferred to a bijou tube (Sterilin) and chilled on ice for 5 minutes. Five micrograms of each RNA transcript GBVC, HCV, MNV, BV, PV and TMEV, were added to each bijou, mixed, and then transferred to a chilled electroporation cuvette (Bio-Rad). The cells were electroporated by using the following settings on a Gene Pulser Xcell (Bio-Rad) eletroporator on the pre-set Mammalian 3 setting: pulse type – exponential decay; capacitance - 500 µF; PC - ∞ Ω; voltage – 160 V; cuvette – 0.4 cm; cell volume – 100 µl. Cells were then transferred to a 12 well plate at 2 x 10^5 cell per well. Cells were collected at 4 hours (h), 24 h, 48 h, 72 h and 96 h. The cells were washed with PBS before being trypsinised with 100 µl of trypsin to remove the cells. The cells were spun at 478 x g for 5 minutes, the supernatant discarded and the cell pellets frozen at -80°C.

2.8.2 Extraction of total cellular RNA

Total cellular RNA was extracted using the RNEasy Mini Kit (Qiagen). Briefly, before starting 2-mercaptoethanol (2-ME) (Sigma) was added to Buffer RLT at a ratio of 10 µl 2-ME in 1 ml RLT to denature cellular RNases. The cell pellets were defrosted at room temperature then disrupted by the adding of 350 µl of Buffer RLT and mixing by pipetting. The disrupted cells were transferred into a QIAShredder (Qiagen) and centrifuged at full speed for 2 minutes. The volume of the lysate was measured and the same volume of 70 % ethanol (VWR) was mixed with it. This was transferred to the RNEasy spin column and either the spin protocol or vacuum protocol followed. In both cases the lysate was passed through the column, the column was then washed with 700 µl Buffer RW1 and

two washes with 500 µl of Buffer RPE. The column membrane was then dried by centrifugation at 8000 x g for 2 minutes. The RNA was eluted in 40 µl of RNase free water by centrifugation at 8000 x g for 1 minute. RNAs were stored at -80°C. Reverse transcription was carried as described previously.

2.8.3 Quantitative real-time PCR

Quantitative real-time PCR is a technique very similar to standard PCR in which specific regions of DNA are amplified by the annealing of specific primers to sense and antisense strands of DNA followed by extension by Taq polymerase so that a specific product is generated. In order to visualise the generation of product the dye SYBR Green is used which binds to double stranded DNA and fluoresces brightly at λ = 522 nm. The real-time machine (Corbett Research) is able to detect the level of fluorescence at each cycle of the PCR reaction which is recorded by computer software. The software plots a graph of fluorescence against cycle number. Analysis of data after the run has finished enables the user to determine the cycle number at which production of specific product has become exponential by crossing a threshold, giving a Ct value. In order to quantify DNA concentrations in samples, standard curves are made consisting of serial dilutions against which sample concentrations can be calculated.

The following reaction mix was assembled including a reaction buffer (Roche) (containing 500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3), 3 mM dNTP, primers 10 OD, FastStart Taq DNA polymerase (Roche) and SYBR Green I (Invitrogen) diluted 1:1000 in DMSO (Sigma).

Nuclease free water	-	14 µl
Buffer, 10x conc with 20 mM $MgCl_2$	-	2 µl
dNTP, 3 mM	-	0.4 µl
Sense primer, 10 OD	-	0.2 µl
Antisense primer, 10 OD	-	0.2 µl
SYBR Green	-	1 µl
FastStart Taq DNA Polymerase	-	0.2 µl
Template	-	2 µl

Primers were designed, for each of the viruses used to make the RNA transcripts, to result in a short product, 100-250 base pairs, which is the optimal size in real-time.

Name	**Sense**	**Start**	**Sequence**	**Product length**
GBV-C	S	131	AGGGTTGGTAGGTCGTAAATCC	235
	AS	366	TGGTCAAGAGAGACATTGAAGG	
HCV	S	339	CCATGAGCACAAATCCTAAACC	163
	AS	502	CTCCGAAGTTTTCCTTGTCGTG	
MNV caps	S	5275	ACCCCAGGTGAAATACTGTTTG	177
	AS	5452	TGGGAAAATAGGGTGGTACAAG	
E11 UTR	S	302	CAACCCCAGTGTAGATCAGGTC	179
	AS	481	AGTTAGGATTAGCCGCATTCAG	
TMEV UTR	S	545	AAGACAGGGTTATCTTCACTCC	217
	AS	762	TGCCTTTGTGTGGTCGCTACAG	

Table 2.13 Primers used in RNA stability study

The following program was set.

No. of cycles	**Temperature (°C)**	**Time**
1	95	2 min
40	94 58 72	25 s 20 s 30 s
1	94	20 s
Melt	Ramps from 65-94 0.3° each step	20 s 1st step 1 s per step for remaining steps

Table 2.14 Condition used in real-time PCR.

2.8.4 Creation of standard curves for stability study

Each of the transcripts used in the study was normalised to 1 µg / µl which was designated as concentration 10^0. Serial ten-fold dilutions in 0.25 µg / ml DNA from herring testes (Sigma) were then made from $10^{-1} – 10^{-9}$. These RNAs then underwent reverse transcription, 5 µl RNA in 20µl reaction volume under previously described conditions. Standard curves were created by running each dilution in duplicate on the real-time PCR machine which also established the limit of detection. During experiments standards were run alongside sample cDNAs. As the sample total cellular RNAs had been treated in an identical way it was possible to determine the concentration of each viral RNA transcript present at each time-point.

2.9 Four kilobase transcripts

2.9.1 Creation of 4 kb RNA transcripts

The interferon induction study consisted of transfecting RNA transcripts into cells and measuring induction of interferon beta (IFN-β) by quantitative real-time PCR indicating the level of IFN-β mRNA present in the cell. These experiments were carried out using the long (full length or truncated) transcripts made by Dr Matthew Davis as previously described, and 4 kilobase (kb) transcripts. These 4 kb transcripts were made to establish that any differing ability of GORS and non-GORS viruses was due to their tendency to form secondary structure alone, and not as a consequence of transcript length or of proteins translated from the RNAs after transfection. The long transcripts were of differing lengths and some of them contained IRES's within their 5' UTRs: GBVC, HCV, PV, and TMEV. The 4 kb transcripts were made by designing primers to amplify a region of the genome beyond the 5' UTR of those viruses containing one. The viruses not containing a UTR included were MNV, BV, SFV and PEMV2. The sense primers contained the T7 promoter sequence at the 5' end to enable transcription of the PCR product into RNA.

Name	Position	Product size	Sequence (T7 sequence in bold)
T7-HCV S	T7+594	4028	***TAATACGACTCACTATAGGGG***GCCCCTATATGGGAATGAG
HCV AS	4622		AGACGTCCAACCCTCTATAGTATGC
T7-GBVC S	T7+563	4009	***TAATACGACTCACTATAGGG***TCCTTCTGCTCCTTCTCGTG
GBVC AS	4572		GGGAGATGGTAATGGTGGGATCAAG
T7-MNV S	T7+147	3943	***TAATACGACTCACTATAGGG***TTACATGACCCCTCCTGAGC
MNV AS	4090		TTTATCTTCCCGTAGATCTTGTCTG
T7-BV S	T7+165	4083	***TAATACGACTCACTATAGGG***TATTTTGGTCGGGAGCTTTG
BV AS	4248		TACTTCGCATATCACTTGTTTCTCC
T7-PV S	T7+850	3938	***TAATACGACTCACTATAGGG***TCAGCTAGTAACGCGGCTTC
PV AS	4788		GTGGATGCTAGAACGTAATTTGAAG
T7-SFV S	T7+167	4112	***TAATACGACTCACTATAGGG***AGGTGGAGTCATTGCAGGTC
SFV AS	4279		ATGACTGTTTTAATTGTGCCCACTG
T7-TMEV S	T7+1163	4000	***TAATACGACTCACTATAGGG***ATGGTTCCCTACGGACCTTC
TMEV AS	5163		TTTGTCCTAGATCATCCATAATCAC
T7-PEMV S	T7+200	4022	***TAATACGACTCACTATAGGG***GGAGTGCAACCTCCTTATCC
PEMV AS	4222		AGGGAGGAAGAAGCACAACCGGTCC

Table 2.15 Primers used to create 4 kb transcripts, with T7 sequence attached to sense primers

2.9.2 Four kilobase PCR

The 4 kb PCR products were made from 100 ng of the full length clones; the following reaction mix was prepared:

Nuclease free water	-	37.2 µl
5x Colourless GoTaq Reaction Buffer	-	10 µl
dNTPs, 3 mM	-	0.8 µl
Sense primer, 10 OD	-	0.4 µl
Antisense primer, 10 OD	-	0.4 µl
GoTaq DNA Polymerase, 5 U / µl	-	0.2 µl
Template, 100 ng (plasmid 100ng / µl)	-	1 µl

The PCR reaction was run under the following conditions:

No. of cycles	Temperature (°C)	Time
1	95	2 min
30	95 50 72	30 s 30 s 5 min
1	72	5 min

Table 2.16 4 kb PCR conditions

Ten microlitres of the PCR products were run on a 1 % agarose gel to check that each band was a single band of the correct size.

2.9.3 Gel purification of 4 kb PCR products

The PCR products were gel extracted to isolate the 4 kb product. This minimised the risk that there would be any contamination of the subsequent *in vitro* transcription reaction with the original template plasmid, ensuring that only the 4 kb product would be transcribed into a 4 kb RNA transcript. The remainder of the PCR products, 40 µl, were run in a 1 % agarose gel for 1 hour to allow the bands to move apart making them easier to identify and cut. The bands were cut using a clean scalpel blade and placed in 1.5 ml tubes (Eppendorf). The gel extraction was carried out using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Briefly, 600 µl buffer QG was added to the tubes containing the gel fragments, they were incubated at 50°C for 10 minutes with frequent mixing by vortexing until the gels had dissolved. One volume of isopropanol (Sigma-Aldrich) was mixed with the samples and added to the QIAquick spin columns. The columns were centrifuged at 17900 x g and the flow-through discarded. A further 0.5 ml of Buffer QG was added to the column which was spun again, then 0.75 ml of Buffer PE was added to the column which was spun again. The flow-through was discarded and the column spun again for another 1 minute to completely remove residual buffers from the membrane. DNA was eluted in 30-40 µl of Buffer EB. Gel extracted products were run on a 1 % agarose gel to ensure the 4 kb products had been preserved in the eluted DNA.

2.9.4 Second PCR and PCR purification

The gel extracted 4 kb PCR products were then used as the template for a second round of PCR using the same sets of primers under the same conditions to increase the yield. The PCR product was run on a 1 % agarose gel. In order to create a clean product to be used in the *in vitro* transcription reaction the PCR product was purified by using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. Briefly, 5 volumes of Buffer PB were added to the PCR samples and mixed and transferred to the QIAquick columns. The columns were centrifuged at 17900 x g for 1 minute and the flow-through discarded. The column was washed by adding 0.75 ml of Buffer PE and centrifuged for 1 minute; the column was centrifuged for a further minute to remove any residual buffers. The DNA was eluted in 30-50 µl of Buffer EB. The eluted DNA was run on a 1 % agarose gel.

2.9.5 DNA Precipitation

In order to concentrate the DNA further it was precipitated in ethanol. Two volumes of ethanol, 0.1 x volumes of 5M sodium chloride (NaCl) and 1 µl of 20 mg /ml glycogen (Roche) were added to the DNA. The DNA was precipitated on ice on 30 minutes then centrifuged at full speed on a bench top centrifuge at 4°C for 30 minutes. The supernatant was removed and 500 µl of 70 % ethanol added to the tube containing the pellet. The tube was centrifuged at full speed for 15 minutes at 4°C. The supernatant was removed and the pellet air dried at room temperature for 5 minutes. The DNA was resuspended in 12 µl nuclease free water.

2.9.6 Determination of nucleic acid concentration

Concentration of DNA or RNA was determined by using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Absorbance measured at 260 nm was used to calculate DNA / RNA concentration expressed in ng / µl.

2.9.7 *In vitro* transcription

In vitro transcription was carried out using the Megascript T7 kit (Ambion) following the manufacturer's protocol. Briefly, a 20 µl reaction was assembled consisting of 2 µl of each of the 4 nucleotides solutions (ATP, CTP, GTP, UTP), 2 µl 10x Reaction Buffer and 2 µl of Enzyme Mix. A mass of 500 – 800 ng of the purified PCR product containing the T7 promoter sequence was added in a volume of 8 µl of nuclease free water. The reaction was incubated for 5 hours or overnight at 37°C. After the incubation 1 µl of TURBO DNAse was added to the reaction mix and incubated at 37°C for 15 minutes to remove template DNA.

2.9.8 RNA Cleanup

The RNA was cleaned up using the RNEasy Mini Kit (Qiagen) following the manufacturer's RNA Cleanup protocol. Briefly, the RNA solution was made up to 100 µl with nuclease-free water; and 350 µl Buffer RLT added. To the RNA was added 250 µl 100 % ethanol and mixed, the sample was then transferred to the RNEasy Mini spin column. The column was centrifuged at 8000 x g for 15 seconds (s) and the flow-through discarded. The column was washed by adding 500 µl Buffer RPE and centrifuging for 15 s followed by a second wash and centrifugation for 2 min. RNA was eluted in 40 µl nuclease free water. RNA concentration was determined by spectroscopy.

2.9.9 Calculation of RNA copy number

RNA copy numbers were calculated by using the following equations based on a single nucleotide of RNA having a molecular mass of 330 and a 4 kb transcript length:

Transcript length x 330 = transcript molecular mass

$4000 \times 330 = 1.32 \times 10^6$

Therefore 1 mole of 4 kb transcript has a mass of 1.32×10^6 g.

$$Mass\,1\,copy = \frac{Mass\,1\,mole}{6x10^{23}}$$

$$Mass\,1\,copy = \frac{1.32x10^{6}}{6x10^{23}} = 2.2x10^{-18}\,g$$

The same equation was used to calculate the mass of a single copy of the full length or truncated transcripts according to transcript length. The mass of single copies of DNA molecules e.g. PCR products was determined using the same equation based on a single DNA base pair having a molecular mass of 650.

2.9.10 Phosphatase treatment

To remove 5' phosphate groups from the RNA transcript a phosphatase treatment was carried out using Antarctic Phosphatase (New England Biolabs). One µg of RNA was mixed with 1 µg of 10 x Reaction Buffer and 1 µl of enzyme in 10 µl reaction volume. The reaction was incubated at 37°C for 15 minutes then inactivated at 65°C for 5 minutes. The RNAs were then visualised on an agarose gel to ensure they had not degraded during the phosphatase treatment.

2.10 Interferon induction study

2.10.1 Transfection of RNA transcripts

Twelve well plates were prepared with NIH3T3 cells and A549n cells at a seeding density of 1 x 10^5 cells per well. The RNA transcripts to be transfected were diluted to the appropriate concentrations and kept on ice. Lipofectamine 2000 (Invitrogen) was added to GIBCO Opti-MEM (Invitrogen) at a ratio of 1-2 µl Lipofectamine 2000 mixed in 100 µl Opti-MEM per transfection. The RNA transcripts were also diluted in 100 µl Opti-MEM per transfection and incubated for 5 minutes. The RNA transcripts were mixed with the diluted Lipofectamine 2000 and incubated at room temperature for 20 minutes. During the incubation the cell culture medium was removed from the cells and replaced with 0.5 ml of 2 % FCS medium. After the incubation 200 µl of the complexes were added to the cells and the plates rocked gently by hand. The plates were returned to the 37°C incubator and incubated for 18 hours. At the end of incubation the cells were removed, trypsinised and pelletted by centrifugation at 478 x g at room temperature for 5 minutes. The cell pellets were stored at -20°C or -80°C. Total cellular RNA was extracted using the RNEasy Minikit as previously described. The RNA concentration was determined by spectrophotometry using the NanoDrop 1000 as previously described. For each experiment the same mass of RNA (500 ng – 1 µg) was used as a template for reverse transcription into cDNA. Reverse transcription reaction conditions were the same as previously described.

2.10.2 Quantitative real-time PCR of IFN-β and 18SrRNA

Primers were designed for use in quantitative real-time PCR to detect murine IFN-β and 18S rRNA. 18S rRNA was used as the housekeeping gene as an internal standard as it has been recommended for use in mRNA quantification (Thellin *et al*., 1999).

Name	Sense	Start	Sequence	Product length
Murine IFN-β	S	71	CACAGCCCTCTCCATCAACT	151
	AS	222	GCATCTTCTCCGTCATCTCC	
18SrRNA	S	1577	GTAACCCGTTGAACCCCATTC	151
	AS	1728	ACCATCCAATCGGTAGTAGCG	

Table 2.17 Primers for murine IFN-β and 18SrRNA PCRs

Name	Sense	Start	Sequence	Product length
Homo sap IFN-β	S	46	GTAGTAGGCGACACTGTTCGTG	491
Homo sap IFN-β	AS	537	GTGACTGTACTCCTTGGCCTTC	
Homo sap IFN-β	S	267	GAACTTTGACATCCCTGAGGAG	137
Homo sap IFN-β	AS	404	GCCAGGAGGTTCTCAACAATAG	

Table 2.17 Primes for human IFN-β PCR.

For use in a quantitative real-time PCR it was necessary to create a standard curve. NIH3T3 cells were infected with Semliki Forest virus at moi 10 as it is able to induce an interferon response. When the cells were showing CPE, but before widespread cell death, the cells were collected and spun down. The total cellular RNA was extracted using the RNEasy Minikit then cDNA made from 5 µl RNA using random primers in a reverse transcription reaction. A PCR was undertaken under standard conditions from 2 µl cDNA in a 20 µl reaction. The PCR product was run on an agarose gel to ensure a single product of the correct size. The remainder of the PCR product was purified using the QIAquick PCR purification kit. The DNA concentration was determined by spectrophotometry.

The copy number of each PCR product was then determined based on a DNA base pair having a molecular mass of 650; IFN-β and 18S rRNA PCR product lengths were both 151 bp.

PCR product length x 650 = molecular mass

$151 \times 330 = 9.815 \times 10^4$

Therefore 1 mole of IFN-β PCR and 18SrRNA product have a mass of 9.815 x 10^4 g.

$$Mass\,1\,copy = \frac{Mass\,1\,mole}{6x10^{23}}$$

$$Mass\,1\,copy = \frac{9.82x10^{4}}{6x10^{23}} = 1.64x10^{-19}\,g$$

Therefore, 1 ng PCR product is equivalent to 6.1 x 10^9 copies; 1 pg PCR product is equivalent to 6.1 x 10^6 copies. Samples for standard curves were made by making serial ten-fold dilutions of these PCR products in 0.25 µg / ml herring testis DNA. The real-time PCR reaction mix was made up as previously described. The results were then expressed as IFN-β cDNA copy number per 1000 copies of 18S rRNA cDNA.

2.10.3 Luciferase reporter assay

NIH3T3 or A549 cells were seeded into 24 well plates, as for the IFN induction experiments as described above, but the level of IFN induction was measured by the expression using the Dual-Luciferase Reporter Assay System (Promega). The luciferase plasmid were transfected by Lipofectamine 2000 (Invitrogen) as described above; *p125-Luc* (firefly) at 100 ng per well; *pRL-CMV* (renilla) at 50 ng per well; 24 hours post-transfection of the luciferase plasmids, the RNA transcripts were transfected as above. 18 hours post-transfection, the cells were lysed with 100 µl passive lysis buffer per well; the lysates were analysed

immediately or the plates were frozen. Seventy microlitres of Luciferase Assay Reagent II (LARII) predispensed into eppendorf tubes for each sample. Ten microlitres of each lysate were added to the tubes and placed in the Glomax 20/20 luminometer (Promega), programmed for the dual-luciferase assay, and the firefly activity measured. A further 70 µl of Stop & Glo Reagent was added to the tubes and the renilla activity measured.

Chapter 3 Equine Rhinitis A Virus – prevalence in Scottish horses

Chapter 3 Equine Rhinitis A Virus (ERAV)

3.1 Introduction

Equine rhinitis A virus (ERAV), a virus predicted to have a genome with GORS, was the first virus chosen to study the possible roles of GORS. It is a member of the A*phthovirus* genus of the *Picornaviridae* family.

3.1.1 Picornaviridae

Picornaviruses are nonenveloped, single-stranded, positive sense RNA viruses. The family *Picornaviridae* consists of the following 12 genera:

i) *Enterovirus*	e.g. human enterovirus, human rhinovirus
ii Cardiovirus	e.g. Theiler's murine encephalomyelitis virus
iii) Aphthovirus	e.g. foot-and-mouth disease virus, equine rhinitis virus
iv) Hepatovirus	e.g. hepatitis A virus
v) Parechovirus	e.g. human parechovirus
vi) Erbovirus	e.g. equine rhinitis B virus
vii) Kobuvirus	e.g. Aichi virus
viii) Teschovirus	e.g. porcine teschovirus
ix) Sapelovirus	e.g. porcine sapelovirus
x) Senecavirus	e.g. Seneca Valley virus
xi) Tremovirus	e.g. avian encephalomyelitis virus
xii) Avihepatovirus	e.g. duck hepatitis A virus

3.1.1.1 Virion structure

Picornavirus particles are spherical, diameter of ~30 nm, consisting of a protein capsid surrounding a naked RNA genome. Cardioviruses, enteroviruses, hepatoviruses and parechoviruses are acid stable and retain infectivity at pH lower than 3, whereas rhinoviruses and aphthoviruses are acid labile at less than pH 6. Members of the latter two genera are usually found replicating in the respiratory system so there is no requirement to be able to survive the acidic conditions present in the stomach (Racaniello; Fundamental Virology, 4th Edition, Edited by Knipe and Howley, Chapter 18, 529-567). The capsid consists of four structural proteins: VP1, VP2, VP3 and VP4. These subunits form an icosohedral structure consisting of 60 subunits (Caspar and Klug, 1962; Rueckert *et al*., 1969). The building block of the capsid is the protomer consisting of one copy each of proteins VP1-3; VP4 is an internal protein. The surfaces of picornavirus capsid are variable; *poliovirus* and *rhinovirus* have prominent plateaus and canyons, whereas *aphthoviruses* and *cardioviruses* are smooth. These features determine which structures interact with a variety of host cell receptors. The RNA genome is not thought to completely fill the space within the capsid. Myristic acid is a residue linked to VP4 on the interior of the virion and has a role in virus assembly and stability of the capsid (Chow *et al*., 1987).

3.1.1.2 Genome structure

The picornavirus genome is a positive-sense, single-stranded RNA molecule so may be directly translated by the cellular machinery. The 5' end of the genome is covalently linked to a protein, the VPg (virion protein, genome linked) (Flanegan *et al*., 1977; Lee *et al*., 1977). The VPg of FMDV is encoded by three genes, although it is encoded by a single gene in other picornaviruses (Forss *et al*., 1982). At the 5' untranslated regions (UTR) are long (100-170 nucleotides in aphthoviruses) and contain highly structured elements. The internal ribosome entry site (IRES) allows translation to occur by binding to the cell's ribosome. There are four types of IRES, types I, II, III and IV. *Aphthoviruses* and *cardioviruses* have a type II IRES immediately downstream of a poly (C) tract. The IRES consists of a structured region predicted to contain

two to four pseudoknots (Escarmis *et al*., 1995), followed by the cis-acting replication element (CRE) (Mason *et al*., 2002), then the IRES which contains the stem-loops numbered 2-5 (Martinez-Salas *et al*., 1993). The 3' UTR is short but also contains structured elements, often a pseudoknot, which is thought to have a role in viral RNA synthesis (Jacobson *et al*., 1993). Without the 3' UTR the viruses are still infectious, but their infectivity is reduced and they replicate poorly (Todd *et al*., 1997).

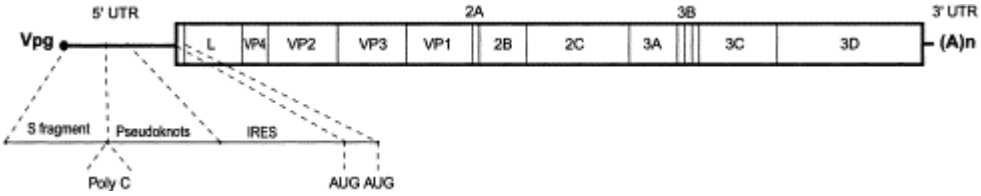


Figure 3.1: FMDV genome taken from Domingo *et al*., 2002

3.1.1.3 Attachment and cell entry of aphthoviruses

Aphthoviruses do not have prominent peaks or canyons, but the βG-βH loop of the FMDV capsid protein VP1 attaches to cellular integrin receptors (Fox *et al*., 1989). There is also a heparan sulphate binding site on FMDV particles in a shallow depression on the surface of the virus (Fry *et al*. 1999). Aphthovirus cell entry is achieved by receptor-mediated endocytosis, FMDV via a clathrin-mediated mechanism (O'Donnell *et al*., 2008) followed by acid dependant uncoating (Carrillo *et al*., 1984; Carrillo *et al*., 1985). At acidic pH, the FMDV capsid separates into pentamers, releasing viral RNA (van Vlijmen *et al*. 1998). . Picornaviruses use many cell surface molecules as receptors; ERAV interacts with sialic acid as its receptor (Stevenson *et al*., 2004).

3.1.1.4 Translation of Picornavirus RNA

Picornavirus positive-sense RNA is translated within the cell cytoplasm. The viral genomes have no 5' cap structure as they have the VPg protein. The process of synthesis of plus and minus RNA strands during replication is initiated by uridylation of VPg (Paul *et al*., 2000) A cellular enzyme unlinks the

VPg (Ambros *et al*., 1980). All picornaviruses contain 5' untranslated regions containing regions of ordered secondary structure (Rivera *et al*., 1988; Skinner *et al*., 1989) which are the IRESes and replication elements. IRES mediated translation is stimulated by an interaction between the structured RNA and elements of the cellular translation machinery. In aphthoviruses, which have a type II IRES, the 3' end of the IRES interacts with eIF4G, eIF4B, eIF3 and PTB (Kolupaeva *et al*., 2003). Translation of the RNA genome from a single open reading frame (ORF) produces a single polyprotein. The polyprotein is divided into three regions: P1, P2 and P3. Aphthoviruses and cardioviruses also code a leader protein (L) in front of P1. A property of the FMDV L^{pro} is the ability to cleave eIF4G which inhibits cellular translation (Devaney *et al*., 1988). The single polyprotein is then cleaved by virus encoded proteinases. The cleavage begins immediately after translation so the polyprotein does not exist in the cell as a single structure. P1 is cleaved to produce the viral capsid proteins; P2 and P3 are cleaved to create the non-structural proteins involved in protein processing and genome replication. In aphthoviruses the VP1/2A cleavage is carried out by $3C^{pro}$ rather than 2A as in other picornaviruses (Palmenberg *et al*., 1992). The protein $3C^{pro}$ also carries out the cleavage between 2C and 3A. Aphthovirus protein 2A, although only 18 amino acids long, is still able to catalyze its autocleavage from 2B (Ryan and Drew, 1994). The proteases initially cleave at intramolecular sites (in *cis*), but latterly also act at equivalent sites on other newly translated molecules (in *trans*). In many picornaviruses there have also been identified *cis*-acting replication elements (CRE). These are structured regions found within the genome at various locations in different viruses, some in coding regions and others in non-coding regions; these elements are necessary for replication (Goodfellow *et al*., 2000; Mason *et al* 2002). The *cis*-acting replication element is necessary for synthesis of the negative sense strand (Goodfellow *et al*., 2000) by templating the production of VPg-pUpU (Pathak *et al*., 2007) During genome replication, three forms of viral RNA can exist: i) single stranded RNA; ii) replicative intermediate (RI) – full length RNA from which 6-8 strands are attached; and iii) replicative form (RF) – dsRNA, a full length copy of positive and negative strands. RNA replication has been shown to be associated with smooth membranes called the

replication complex (Girard *et al*., 1967), under the control of the virally encoded enzyme $3D^{pol}$, an RNA-dependant RNA polymerase.

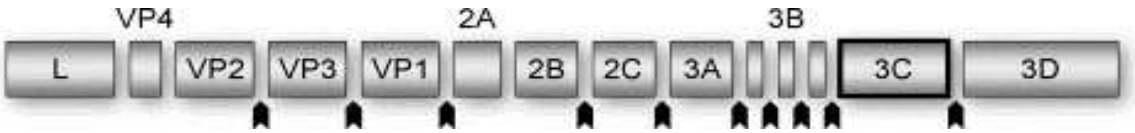


Figure 3.2: FMDV polyprotein including cleavage sites (black arrows) taken from Curry *et al*., 2006

3.1.1.5 Assembly of virus particles

After their initial cleavage, the VP0-VP3 and VP3-VP1 bonds are also cleaved by proteinase $3CD^{pro}$. The capsid proteins, VP0, VP1 and VP3 self-assemble by intra-molecular interactions (Cao *et al*., 2009). Initially five protomers form a pentamer which are important intermediates in the assembly of picornaviruses (Boege *et al*., 1986; Rombaut *et al*., 1990). Pentamers can form into 80S empty capsid-like particles which contain 60 copies of each protein (Kräusslich *et al*., 1990), into which the RNA is inserted to produce provirions (Jacobson *et al*., 1968). The progenitor VP0 protein then cleaves into VP2 and VP4 to form the virion. The final viral particle contains only the positive sense viral RNA because the encapsidation process is very specific, so avoiding inclusion of cellular RNA or negative sense viral RNA (Nomoto *et al*., 1977; Novak and Kirkegaard). *Picornavirus* infections may affect the cells by often inhibiting cellular protein synthesis by inhibiting eIF4G, or they cause cell lysis on extrusion.

3.1.2 Equine Rhinitis A virus

3.1.2.1 Clinical features and epidemiology of ERAV

Equine Rhinitis A virus (ERAV) is a causative agent of upper respiratory tract infection in horses. It was first isolated in the United Kingdom in 1962 (Plummer, 1962) where it was described as an equine respiratory virus with enterovirus properties. Cases have been reported world-wide and the virus

isolated in Canada (Ditchfield and MacPherson, 1965), the United States (Wilson *et al*., 1965), Italy (Flammini and Allegri, 1970), France (Brion 1966), Germany (Becker *et al*, 1974), Switzerland (Hofer *et al*, 1973) and Australia (Studdert and Gleeson 1977) . Young animals in contact with older animals are the most susceptible group (Hofer *et al*, 1972). The incubation period is 3-8 days (Thein, 1978) and there is a viraemic phase of 4-5 days (Plummer and Kerry, 1962). Following aerosol exposure, the virus replicates in the nasal epithelium before establishing viraemia. The virus is cleared from the blood by an antibody response, but it persists in the pharynx. The virus was originally detected in equine faeces (Plummer 1963), although it has not been isolated from intestinal tissue, and it may be detected for up to 4 weeks post-infection. It may be that some viruses present in the pharynx are swallowed and, despite being acid labile, sufficient virions survive to be detected in the faeces. Another possible explanation for faecal detection is that as a consequence of the viraemia the enterocytes become persistently infected. ERAV has been detected in horse urine for up to 146 days (McCollum and Timoney, 1992) so the urinary tract may be another site of persistent infection. Clinical signs include pyrexia (41°C +/- 0.5°C), nasal discharge (initially serous but becoming mucopurulent), coughing, anorexia, pharyngitis and lymphadenitis. Uncomplicated cases usually recover in 7 days, although the horses are susceptible to secondary bacterial infections e.g. *Streptococcus zooepidemicus* (Virus Infections of Equines, Chapter 18, M.J. Studdert). The seroprevalence of antibodies against ERAV in horses in various countries has been determined e.g. 90 % in Austria (Kriegshauser *et al*, 2005), 66 % in the Netherlands (De Boer *et al*, 1979), 79 %, 57 % in USA (McCollum and Timoney, 1992). Horses appear to acquire the infection within the first 2 years of life and antibodies against ERAV are uncommon in animals between 6 and 12 months of age (Studdert and Gleeson, 1978).

3.1.2.2 Classification of ERAV as an aphthovirus

ERAV was previously known as Equine rhinovirus 1 (ERhV1), but its classification was uncertain until Wutz *et al* (1996) and Li *et al* (1996) demonstrated that due to its sequence homology and similar genome organisation it is most closely related to foot-and-mouth disease virus (FMDV), formerly the only member of the genus *Aphthovirus* in the family *Picornaviridae*. The ability to cause a viraemia, persistent infections and broad cell culture host range are also more typical of aphthoviruses than other picornaviruses. The most similar features between ERAV and FMDV include the amino acid sequence of the 2A protein which is 87.5 % identical (Li *et al*, 1996) and a leader (L) protein which is similar in size to the FMDV Lab protein. ERAV and FMDV both have a type II internal ribosome entry site (IRES). Translation of the FMDV polyprotein is initiated from 2 different AUG codons, the first at the 3' end of the IRES and the other 84 nucleotides downstream (Beck *et al*., 1983; Clarke *et al*., 1985) resulting in the production of 2 forms of the leader protein, Lab and Lb (Sangar *et al*., 1987). There are also 2 start codons in ERAV, although they are separated by 54 nucleotides. During translation of the ERAV genome however, the first site appears dominant resulting in an excess of Lab over Lb (Hinton *et al*., 2000), whereas the relative amounts of Lab and Lb varies between FMDV strains (Sangar *et al*., 1987).

3.2 Objectives

1. ERAV prevalence study by serological survey.

In this study equine serum samples were collected from the clinical laboratory of the veterinary pathology unit at the Easter Bush Veterinary Centre, University of Edinburgh. The serological survey was conducted by use of a virus neutralisation assay. Based on reports of the persistence of ERAV infections it was expected that there would have been marked exposure to ERAV in horse in Scotland.

2. ERAV prevalence study by PCR screening of faecal samples and sera

ERAV causes a relatively mild upper respiratory tract infection, so it is unlikely that the animals admitted to the equine hospital would have been referred with respiratory disease due to ERAV as the primary presenting sign. The samples analysed in this study were from inpatients admitted for other reasons, such as orthopaedic or gastrointestinal conditions. As ERAV is a persistent virus, we reasoned that horse may have been exposed to ERAV and recovered clinically but would continue to shed virus. This study sought to determine the level of ERAV detectable in faecal and serum samples.

3. Complete genome sequencing of PCR positive samples for phylogenetic and structural analyses.

Any novel viruses discovered in the clinical samples would then undergo phylogenetic and bioinformatics analysis. Bioinformatic prediction of the degree of secondary structure throughout the genome of ERAV revealed that the 5’ half of the genome was predicted to be structured, but the 3’ half unstructured (data not shown). We hypothesised that this uneven distribution of structure may have been a cell culture adaptation as sequence data are derived from laboratory strains, and that novel ERAVs discovered in clinical samples would contain structure throughout the length of the genome.

3.3 Results

The seroprevalence of anti-ERAV antibodies was established by means of a virus neutralisation assay. The results are summarised in table 3.1. Of 53 equine serum samples, 29 were positive, (55%).

Virus	No. of sera	Neutralisation titre					No. positive	% positive
		0	40	160	640	2560		
ERAV	53	22	2	7	9	11	29	55%

Table 3.1: Neutralisation titres of 53 equine sera.

The same equine sera were also screened for ERAV by PCR. The primers specific for the capsid and the UTR were shown to be sensitive (a ten-fold dilution series of cDNAs was made from RNA extracted from ERAV supernatant at 10^6 $TCID_{50}$/ml; and the capsid primers detected the 10^{-8} dilution, UTR primers detected 10^{-7}; data not shown). .All serum samples were negative as were the fifty equine faecal samples.

PCR – ERAV capsid and screening primers	Equine sera	0/53
	Equine faecal sample	0/50

Table 3.2: Result of prevalence screen of ERAV by PCR of sera and faecal samples

Isolates of ERAV from clinical samples were kindly provided by the Animal Health Trust, Newmarket. These isolates were screened by PCR to ensure the presence of ERAV, and all samples were positive.

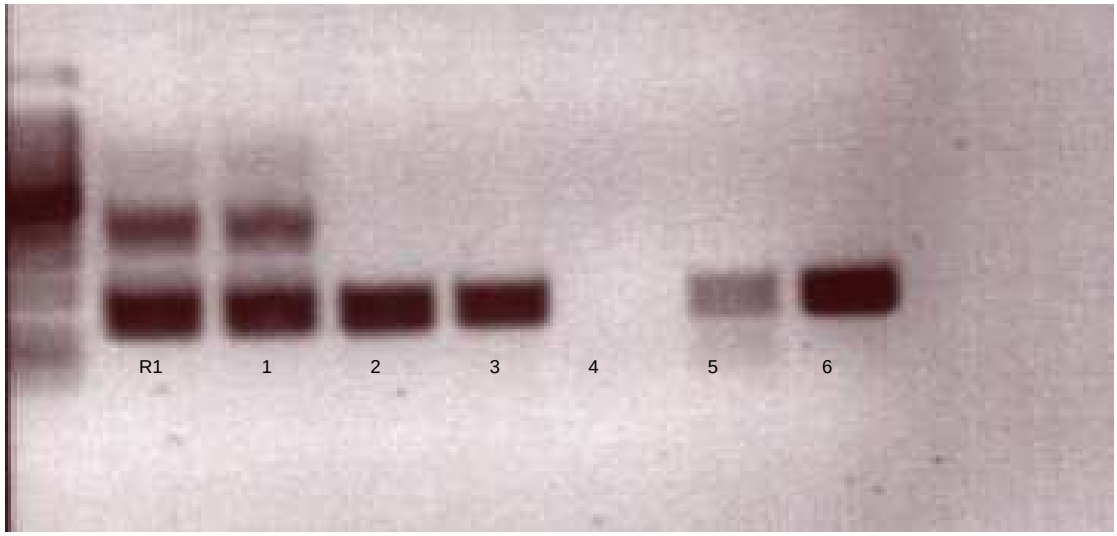


Figure 3.3: Gel showing result of screening with ERAV capsid primers on AHT clinical isolates; samples R1, 1, 2, 3 and 6 were positive with a strong band, R1 and 1 also had a primary product band; sample 5 was a weak band; sample 4 was negative.

AHT clinical samples/isolates	**Sample number**	**PCR result**
R1 isolate	R1	+ve
41185 nasal swab	1	+ve
4590/85 nasal swab	2	+ve
54641 nasal swab	3	+ve
55051 p3	4	-ve
54590 nasal swab	5	+ve
5051/85	6	+ve

Table 3.3: PCR result of AHT ERAV isolates.

As a result of the samples R1 and 1 giving the strongest bands on the gel after the nested PCR these were chosen for complete genome sequencing, sequencing the products of a series of overlapping nested PCRs. The result was that both of these samples had identical sequences to previously published strains of ERAV. Sequences are given in appendix 1.

3.4 Discussion

ERAV, as a virus predicted to have a significant degree of secondary structure over the first half of the genome was a candidate virus for the study of GORS. The study was conducted to determine the prevalence of ERAV in horses in Scotland to test the hypothesis that as viruses predicted to contain GORS are able to form persistent infections (Simmonds *et al*., 2004), and that ERAV has been shown to be shed persistently (McCollum and Timoney 1992), that it would be a highly prevalent. This study has shown a prevalence of antibodies to ERAV of 55 %, which is comparable to that of other countries. The sera submitted were from equine patients at the Equine Hospital, University of Edinburgh, which would have been treated for a wide variety of conditions. The clinical history of the horse corresponding to each serum samples was not available, but as ERAV causes a relatively mild clinical disease it is not likely that the horses referred to the equine hospital would have been clinical case of ERAV. The seroprevalence result of 55 % shows that a majority of horses in Scotland have been exposed to ERAV at some time in their lives. The fact that in this study, no horses of less than 2 years of age were seropositive is also consistent with previous observations of age-related exposure (Studdert and Gleeson, 1978) which showed that 16 % of horses between the ages of 6 and 12 months were seropositive. The screen of each of the positive sera by PCR was negative however, indicating that it is highly unlikely that any of the seropositive horses were viraemic with ERAV.

ERAV was first discovered by Plummer in 1962 in the faeces of 13 out of 290 horses (5 %), it was described as an equine respiratory virus with enterovirus properties. Although it is now known that the virus ERAV is a member of the *Aphthovirus* genus of *Picornaviridae*, and cause an upper respiratory tract infection, it is still not certain how the virus can be detected faecally. Theories include the possibility that the virus from the respiratory tract may be swallowed and so emerge in the faeces, although as an acid-labile aphthovirus it might not be expected to be able to pass through the acidic environment of the stomach. An alternative idea is that as the virus causes a viraemia it could potentially reach any part of the body and therefore be secreted into the intestinal tract. In this study faecal samples were collected from the horse boxes of 50 in-patients

at the Equine Hospital, University of Edinburgh and screened by PCR. None of the faecal samples were positive. The prevalence in faecal samples determined by Plummer i.e. 5 % is markedly below that of the seroprevalence found in this study and others. A faecal prevalence of 5 % in 50 samples would have been given by just 2-3 positive samples, so it may be that a larger sample size was required. A more sensitive detection method may also have revealed hidden positives.

The ERAV isolates provided by the Animal Health Trust, Newmarket, were all detectable by PCR with the exception of one. The almost complete genome sequencing was carried out in an effort to identify a new strain of the virus distinct from the laboratory strains and published sequences. The hypothesis was that newly identified viruses from an animal would be free of cell culture adaptations and related sequence changes that are present in laboratory strains. The hope was that novel virus sequences could be analysed by bioinformatics structural predictions to compare the degree of secondary structure along the length of the ERAV genome. The laboratory strain of ERAV shows regions of predicted secondary structure along the first half (5') of the genome before apparently becoming unstructured towards the 3' end. The plan was then to serially passage the ERAV isolates and assess the effect of cell culture adaptation on the degree of secondary structure along the genome. Unfortunately, none of the clinical samples tested by PCR in this study were positive so there was no novel ERAV isolates could be obtained and the isolates provided by the AHT had identical sequences to the published sequences of the laboratory strains possibly as a consequence of contamination.

This study that the seroprevalence of ERAV in Scotland is comparable to that in other European countries, USA, Canada, Australia and Japan, but it did not prove to be useful virus for the study of GORS.

Chapter 4 Murine norovirus – prevalence, genomic RNA structure and the identification of a novel MNV in the wood mouse (*Apodemus sylvaticus*)

Chapter 4 Murine Noroviruses – prevalence, genomic RNA secondary structure and identification of a novel MNV in the wood mouse (*Apodemus sylvaticus*)

4.1 Introduction

The genus *Norovirus* of the family *Caliciviridae* consists of viruses that infect humans and animals. Human norovirus infections cause mild, acute, self-limiting gastroenteritis characterised by nausea, vomiting, diarrhoea and abdominal cramps (Kaplan *et al*., 1982). Outbreaks typically occur in confined places such as cruise ships, hospitals and schools (Widdowson *et al*., 2005; Becker *et al*., 2000; Gunn *et al*., 1980). Noroviruses have also been discovered in pigs (Sugieda *et al*., 1998), cattle (Woode and Bridger, 1978) and mice (Karst *et al*., 2003). Bovine noroviruses are associated with diarrhoea in calves (Woode and Bridger, 1978) whereas porcine and murine norovirus are subclinical in immunocompetent hosts (Wang *et al*., 2007; Karst *et al*., 2003). Noroviruses are classified into 5 distinct genogroups: genogroups I, II and IV contain human noroviruses, genogroup III contains bovine noroviruses and genogroup V contains murine noroviruses; the genogroups are also divided into genotypes (Green *et al*., 1999); see figure 1.1. Murine noroviruses (MNVs) diverge by up to 13 % at the nucleotide level, and to date are considered to comprise a single genotype of genogroup V (Thackray *et al*., 2007).

Noroviruses have a single-stranded, positive sense RNA genome of ~7.5 kilobases. There are 3 well characterised ORFs. ORF 1 encodes a polyprotein which is cleaved into the non-structural proteins (Sosnovtsev *et al*., 2006). ORF 2, which is sub-genomic, encodes the major capsid protein VP1 (Jiang *et al*., 1990); ORF 3 encodes the small basic protein VP2 which appears to have a role in virion stability (Glass *et al*., 2000). A fourth ORF has been postulated, but is of uncertain function. The 5' end of the genome is covalently bound to the VPg protein and the 3' end is an untranslated region consisting of a stem-loop and a poly(A) tail (Gutierrez-Escolano *et al*., 2003).

MNV was discovered in 2003 in RAG/STAT$^{-/-}$ mice; these mice are severely immunosuppressed and the mice would die with meningoencephalitis, cerebral

vasculitis and pneumonia (Karst *et al*., 2003). The virus was identified as a norovirus and designated MNV-1. MNV-2, 3 and 4 were subsequently characterised by Hsu *et al* in 2006 by screening mesenteric lymph nodes and faecal samples from laboratories across the United States. These three new strains were shown to establish sub-clinical, persistent infections with prolonged faecal shedding, in contrast to MNV-1 (Hsu *et al*., 2006). A further 21 MNVs were sequenced in 2007 which comprised 15 distinct strains with up to 13 % divergence at the nucleotide level (Thackray *et al*., 2007). To date, August 2009, there are 41 MNV complete genomes available on the NCBI Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez).

MNV has also become the favoured virus to study norovirus biology as it is the only norovirus that is cultivatable in cell culture. It was discovered to grow in the murine macrophage cell line RAW 264.7, producing CPE and forming plaques (Wobus *et al*., 2004). This has led to the development of reverse genetics systems by which the virus's replication strategies will certainly be further elucidated in the future (Ward *et al*., 2007; Chaudhry *et al*., 2007).

MNV has also been identified as a GORS virus i.e. prediction of secondary structure along the length of the RNA genome by bioinformatic analysis; the average MFED of MNV-1 being 6.3 %. MNV is an ideal virus for the study of GORS as mice are numerous in research institutions and studies have identified the presence of MNV in laboratory mice in institutions around the world (Hsu *et al*., 2006; Muller *et al*., 2007). The observation that MNV-2, 3 and 4 caused persistent infection in immunocompetent hosts in 2006 agreed with the observation that the presence of GORS is associated with this property (Simmonds *et al*., 2004). The cell culture system and recently developed reverse genetics techniques also allow passage and manipulation of the virus. MNV is a virus that allows us to test hypotheses concerning the nature of GORS and its relationship to persistence in immunocompetent mice.

4.2 Objectives

1. Prevalence study of MNV in laboratory mice and wild mouse populations.

In this study faecal samples were collected from laboratory mice (*Mus musculus*), wild house mice (*Mus musculus*) the following wild rodent species: wood mouse (*Apodemus sylvaticus*), field vole (*Microtus agrestis*) and bank vole (*Myodes glareolus*). The samples were screened by PCR for MNV using specific primers, or degenerate norovirus primers in the wild samples. Based on previous reports, it was expected to identify MNV in laboratory samples; in wild rodents the prevalence of MNV is unknown, but it was hypothesised that a norovirus present in a wild population would likely to be GORS, and as GORS confers an evolutionary advantage in enabling the virus to persist there would be a similar prevalence as in mice bred in captivity.

2. Phylogenetic analysis of novel MNVs

Previous studies have shown that MNV is the only member of norovirus genogroup V, and consists of a single genotype. The aim of our study was to classify MNVs identified in laboratory mice and wild mice. To this end, regions of the genome were sequenced from PCR positive samples, and the complete or almost complete genomes sequenced from several of them.

3. RNA structural predictions

Using the complete or almost complete sequences of MNVs identified as well as selected previously published sequences, the degree of secondary structure was predicted along the length of the genome. It was hypothesised that any newly identified MNVs from laboratory mice or wild animals would all be GORS viruses.

4. MNV passage study

We formed a hypothesis that during infection of an animal, the immune system exerts an evolutionary pressure and that GORS, in some way perhaps related to immune evasion, confers an advantage, so the degree of secondary structure is maintained by the virus. We considered that in cell culture viruses are known to undergo adaptations, and that after serial passage the degree of secondary structure may decline as the virus becomes cell culture adapted. It has been shown that the presence of GORS is sensitive to changes in sequence, 2 % sequence drift reducing the MFED, and 10 % drift undoing the presence of GORS completely. MNV-3 was therefore passaged 33 times in RAW cells at high and low moi; the complete genome of passage zero and 33 were sequenced.

4.3 Results

4.3.1 Prevalence of MNV

Faecal samples from laboratory mice at the University of Edinburgh were kindly collected by Mr Billy Smith. Access to wild rodent samples was kindly provided by Anna Meredith. Other house mouse samples were provided by various members of the Centre for Infectious Diseases at the University of Edinburgh. The pet shop mouse samples were collected from Morningside Pets, Edinburgh. The faecal samples were screened by PCR using a capsid primer The prevalence of MNV in these various populations is summarised in table 4.1

PCR: MNV capsid or degenerate norovirus primers	Laboratory mice	26/39
	Pet shop mice	2/2
	Wild rodents:	
	i) *Mus musculus* (House mouse)	0/15
	ii) *Apodemus sylvaticus* (Wood mouse)	1/11
	iii) *Microtus agrestis* (Field vole)	0/8
	iv) *Myodes glareolus* (Bank vole)	0/12

Table 4.1 Table showing prevalence of MNV in faecal samples from 39 laboratory mice, 2 pet shop mice, 11 wood mice, 8 field voles and 12 bank voles. Samples from *Mus musculus* were screened using MNV specific primers, samples from the other wild rodent species were screened with both MNV specific primers and degenerate norovirus primers.

The MNV positive faecal samples from the laboratory mice were expected, in accordance with previously published studies. The two samples from the pet shop were taken from different cages with a number of animals in each; both samples were PCR positive. The PCR positive sample from the *Apodemus sylvaticus* is to the best of the author's knowledge, the first identification of a norovirus in a wild rodent.

4.3.2 Phylogenetic analysis

After identification of PCR positive samples in the initial screening procedure, two regions of the genome were selected to be sequenced for phylogenetic analysis. The first region, designated L1, produced an amplicon from position 1016 – 1762 in the non-structural region of the genome; the second, designated L2, produced an amplicon from position 6142 – 6882. Using these primer sets

in a PCR on the 26 PCR positive laboratory samples, 17 were positive for L1 and 15 for L2. The complete or almost complete genomes of six novel MNVs were sequenced: 4 laboratory MNVs designated Lab 12, Lab 17, Lab 18, Lab 22; and the pet shop mouse MNV (PSM1), (the MNVs identified from the two pet shop cages had identical sequences); and the *Apodemus* MNV (WR960). The complete sequence data can be found in Appendix 1. Phylogenetic analysis was carried out using Mega 4 (Mega 4.0.1, Tamura, Dudley, Nei and Kumar, 2007). Figure 4.1 shows a tree made from the almost complete nucleotide sequence of the 6 novel MNVs and all other previously published sequences. Figure 4.2 is a tree made from the almost complete amino acid sequences of the 6 novel MNVs and published sequences. What is notable is that the nucleotide and amino acid trees are very similar and that whereas the laboratory MNVs and pet shop MNV cluster closely together, the *Apodemus* MNV does not, and appears to be a significant genetic distance from the others. Phylogenetic trees made from the nucleotide sequences of L1 and L2, figures 4.3 and 4.4, confirm that the majority of laboratory MNVs cluster closely with other published MNV sequences. Figure 4.5 shows a phylogenetic tree made from the amino acid sequence of the VP1 region of selected examples of every genotype of each norovirus genogroup. The position of WR960 clearly shows that it is a member of the MNV genogroup i.e. genogroup V, and therefore not a contaminant from a human, bovine or porcine norovirus.

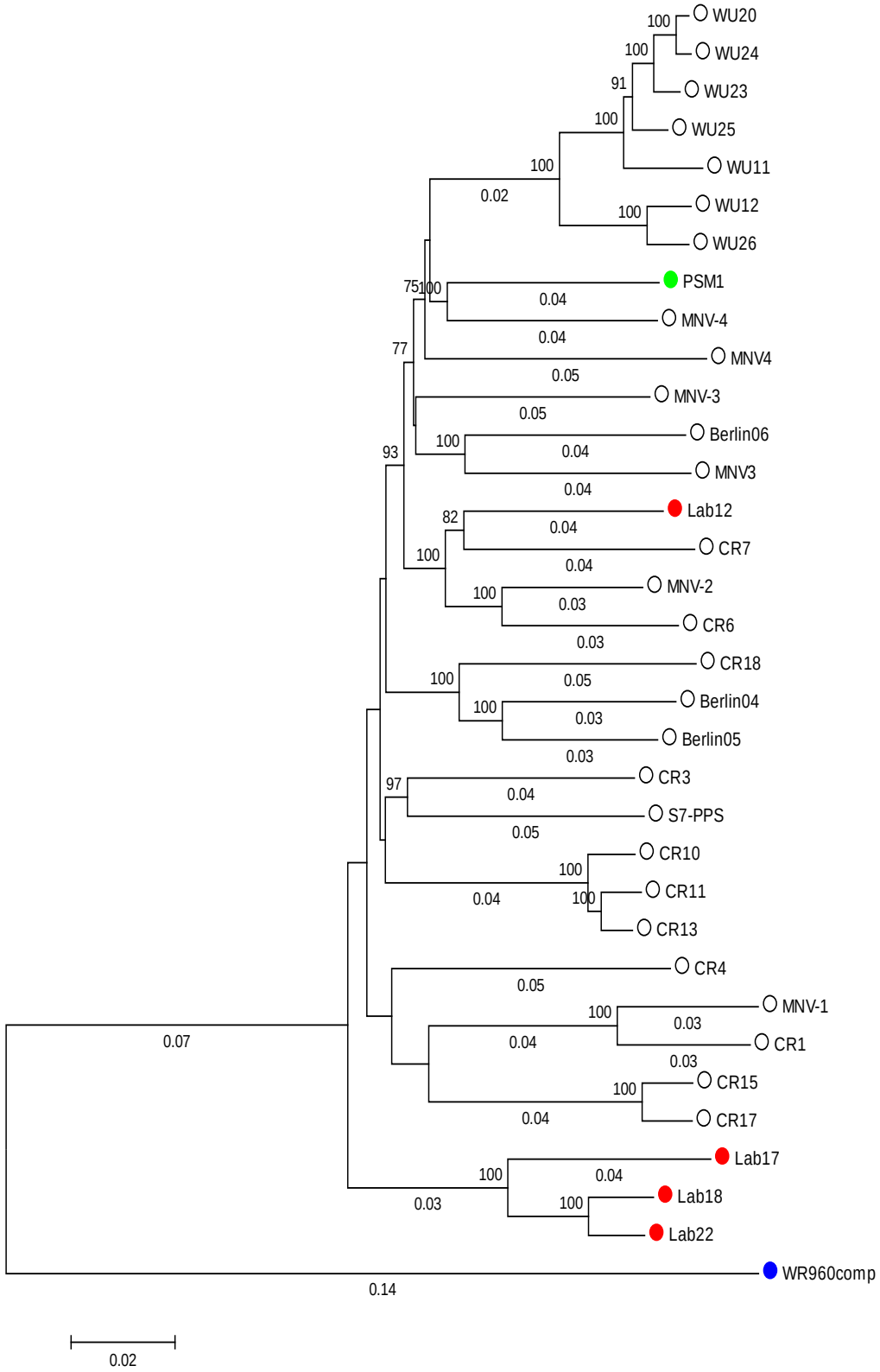


Figure 4.1 Phylogenetic analysis of nucleotide sequence by neighbour joining using the Jukes-Cantor model with pairwise deletion, of almost complete sequences of 6 newly sequenced MNVs and all published sequences from GenBank. Laboratory mouse MNVs are labelled with red dots; pet shop mouse, green; wood mouse, blue.

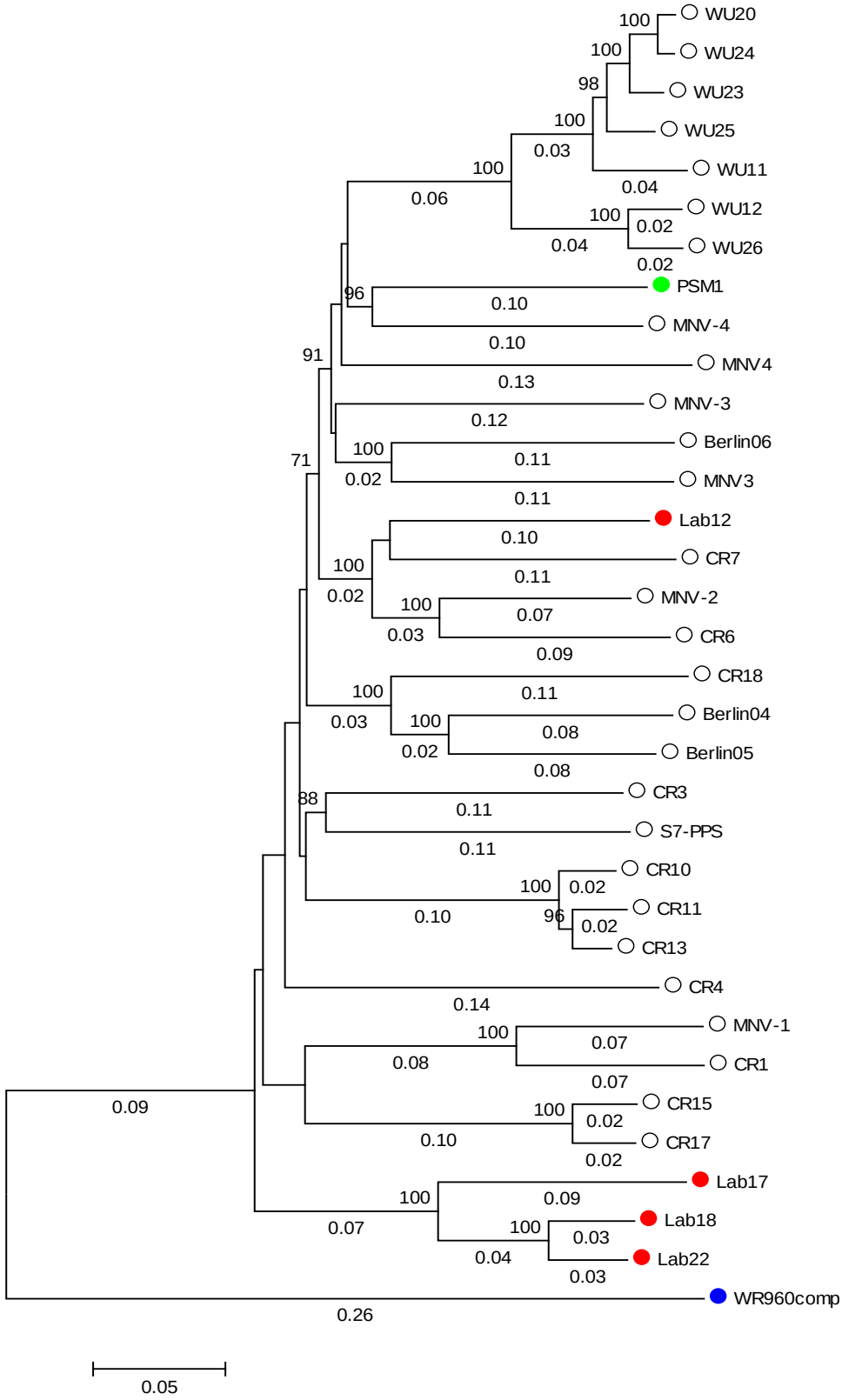


Figure 4.2 Phylogenetic analysis of amino acid sequence using p distance with pairwise deletion of almost complete sequences of 6 newly sequenced MNVs and all published sequences from GenBank. Laboratory mouse MNVs are labelled with red dots; pet shop mouse, green; wood mouse, blue.

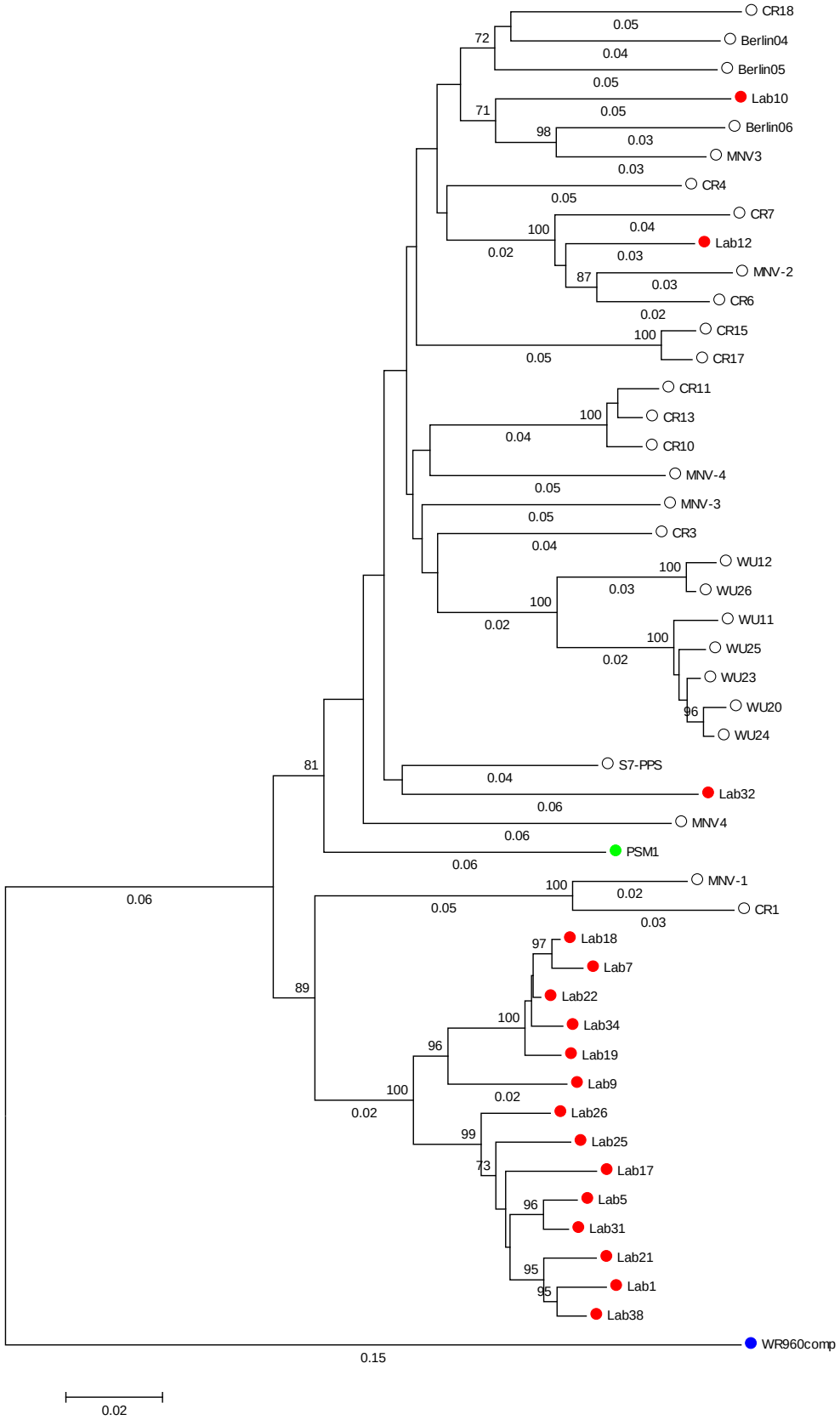


Figure 4.3 Phylogenetic analysis of nucleotide sequence by neighbour joining using the Jukes-Cantor model with pairwise deletion, from position 1016 – 1762, of 19 newly identified MNVs and all published sequences from GenBank. Laboratory mouse MNVs are labelled with red dots; pet shop mouse, green; wood mouse, blue.

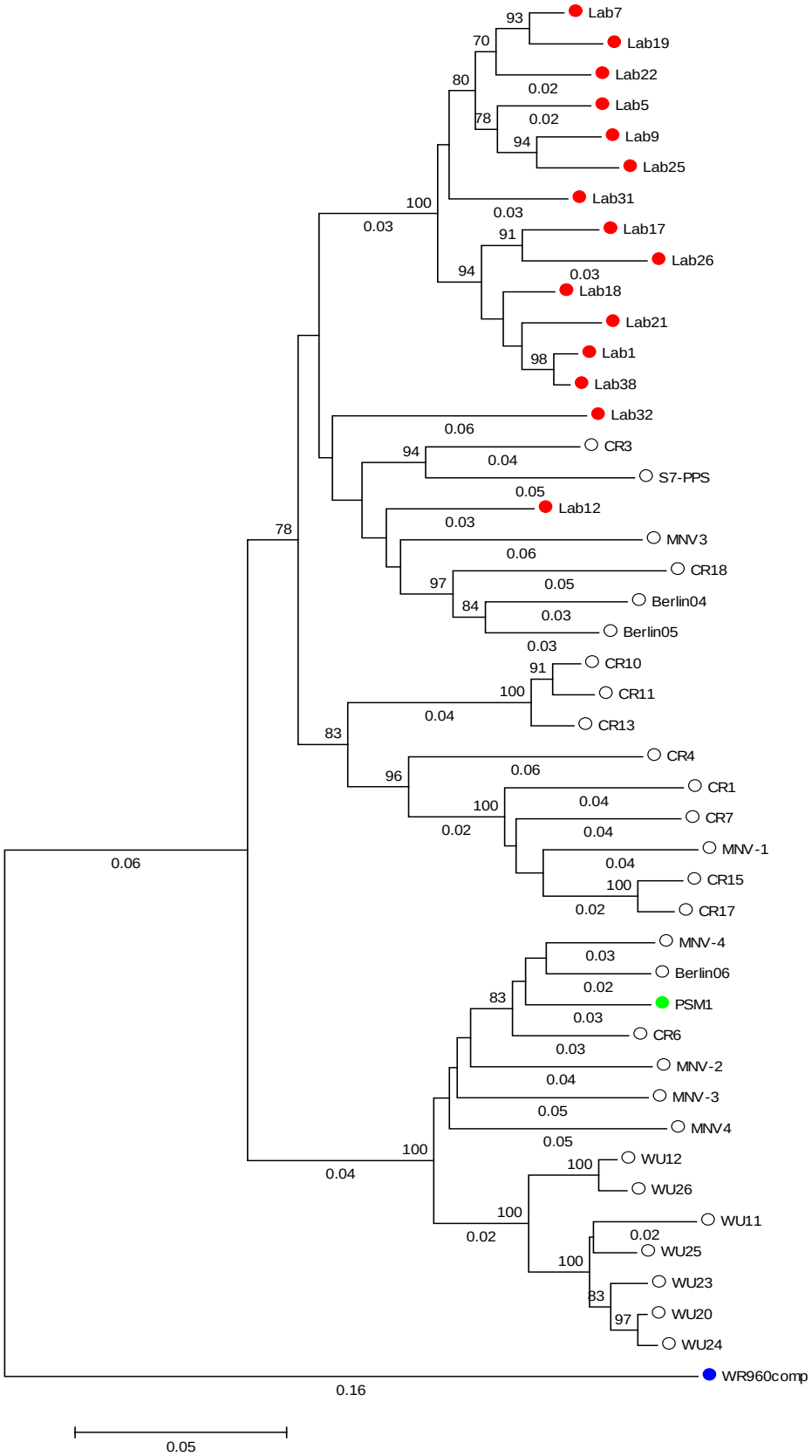


Figure 4.4 Phylogenetic analysis of nucleotide sequence by neighbour joining using the Jukes-Cantor model with pairwise deletion, from position 6142 – 6882, of 17 newly identified MNVs and all published sequences from GenBank. Laboratory mouse MNVs are labelled with red dots; pet shop mouse, green; wood mouse, blue.

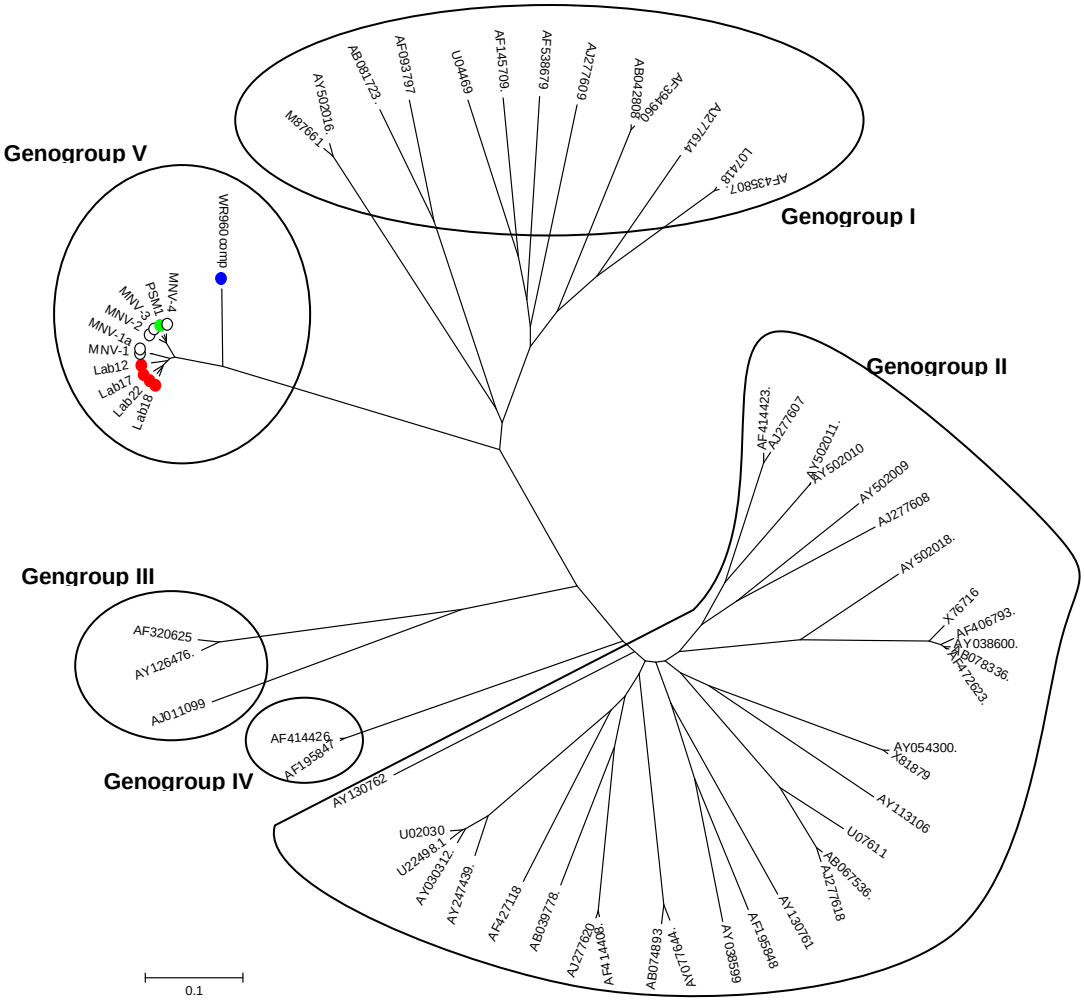


Figure 4.5 Phylogenetic analysis of the amino acid sequence of the capsid protein VP1 with pairwise deletion of 6 newly sequenced MNVs and selected published sequences from GenBank from every norovirus genotype of every genogroup. Laboratory mouse MNVs are labelled with red dots; pet shop mouse, green; wood mouse, blue.

The p distances were calculated using the Simmonics sequence editing package of each of the novel MNVs and the average p distance of the published complete genome sequences. Table 4.2 shows the p distances of the almost complete genome sequence of the 6 newly sequenced MNVs and the average p distance of all other previously published complete MNV sequences. Table 4.3 shows the p distances of the amino acid sequence of the major capsid protein VP1 of the same 6 novel MNVs and the average p distance of all other published MNVs. In both tables the p distances between the wood mouse MNV (WR960) and the other MNVs is higher than the p distance between the other MNVs and the average of the published sequences

WR960	-						
Lab12	**0.27**	-					
Lab17	**0.28**	0.14	-				
Lab18	**0.27**	0.12	0.07	-			
Lab22	**0.27**	0.12	0.07	0.02	-		
PSM1	**0.27**	0.11	0.14	0.12	0.12	-	
Published (average)	**0.27**	0.11	0.14	0.12	0.12	0.11	0.11
	WR960	**Lab12**	**Lab17**	**Lab18**	**Lab22**	**PSM1**	**Published (average)**

Table 4.2 Table showing p distances based on nucleotide sequence of the complete genome from 6 novel MNVs: WR960 (wood mouse MNV), 4 laboratory mouse MNVs, pet shop mouse MNV and the average p distance of published MNVs

WR960	-						
Lab12	**0.16**	-					
Lab17	**0.16**	0.04	-				
Lab18	**0.16**	0.04	0.02	-			
Lab22	**0.16**	0.03	0.02	0.02	-		
PSM1	**0.16**	0.06	0.06	0.05	0.06	-	
Published (average)	**0.16**	0.05	0.05	0.04	0.04	0.04	0.04
	WR960	**Lab12**	**Lab17**	**Lab18**	**Lab22**	**PSM1**	**Published (average)**

Table 4.3 Table showing p distances based on amino acid sequence of VP1 protein from 6 novel MNVs: WR960 (wood mouse MNV), 4 laboratory mouse MNVs, pet shop mouse MNV and the average p distance of published MNVs

4.3.3 MNV secondary structure prediction

There were 26 MNV positive mice identified by PCR in the initial screening of laboratory mice faeces, 2 from pet shop mice and 1 from the wild rodent species, *Apodemus sylvaticus*. Of these, the complete or almost complete genomes were sequenced from 4 laboratory samples, the pet shop sample and the wild rodent sample by use of a series of overlapping primer sets to cover the length of the genome but for the very 5' and 3' ends. The sequences were edited and aligned in the Simmonics v1.7 editing package. Using UNAfold (Markham and Zuker, 2008) with NDR randomisation, secondary structure predictions were made along the length of the genome from position 58 to 7318; 776 fragments of 300 nucleotides were analysed at intervals of 9 nucleotides; the sequence was scrambled using the NDR randomisation. Table 4.3 shows the average MFED across the entire genome of the 10 viruses. The threshold for definition of a GORS virus is generally considered to be 5-6 %. All viruses are predicted to be GORS viruses, although some may be considered borderline e.g. WR960.

	MNV1	MNV2	MNV3	MNV4	Lab12	Lab17	Lab18	Lab22	PSM1	WR960
Average MFED	6.31%	8.52%	7.68%	7.95%	7.46%	8.17%	7.39%	7.11%	6.38%	5.12%

Table 4.3 Average MFED values along the length of almost the entire genome based on the MFED of 776 fragments of 300 nucleotides at intervals of 9 nucleotides from position 58 to 7318.

Figures 4.6 A-J show the average MFED of 11 consecutive fragments of nine bases i.e. the average of fragments of 99-bases, along the length of the genome; the mid-point of every nine-base fragment is therefore plotted against the average of its MFED and the five fragments before and after it on the genome. The columns are positioned at the mid-points of each fragment, the first at position 245, the last at position 7150. Figure 4.7 shows a histogram of MFED against nucleotide position for all fragments. All viruses analysed showed predicted structures along the length of their genomes, so may be characterised as GORS viruses, although throughout the genome there are some unstructured areas.

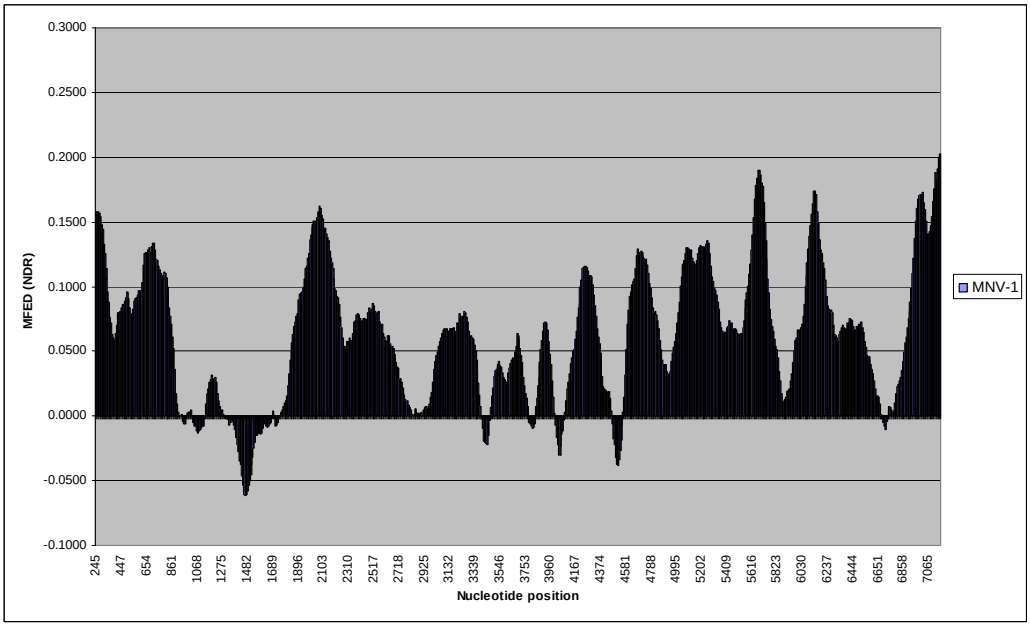


Figure 4.6 A Distribution of MFEDs along the length of genome of MNV-1; mean MFED of sequential 99-base fragments are shown.

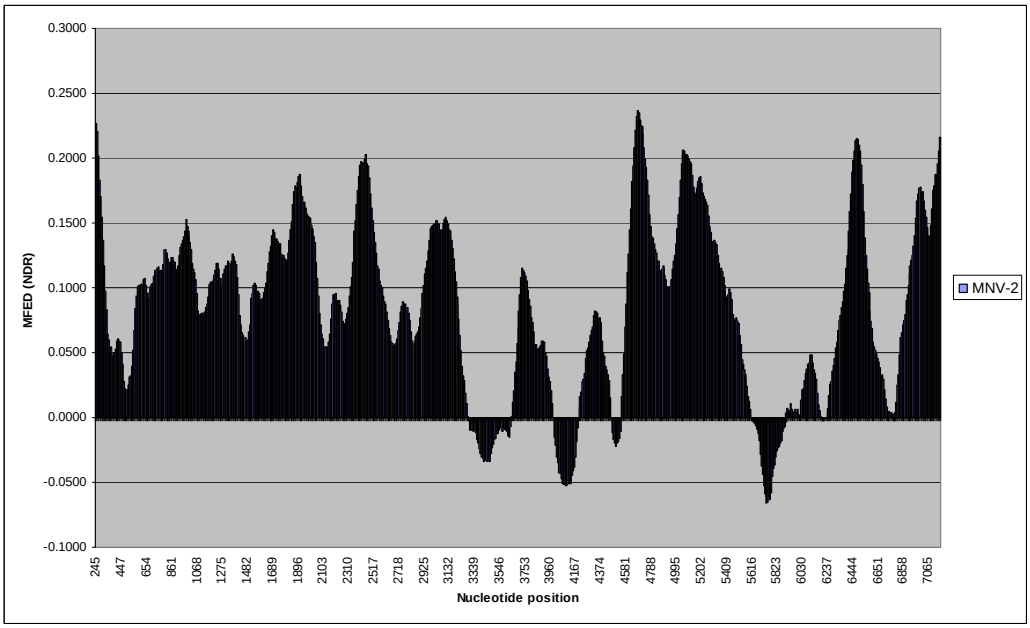


Figure 4.6 B Distribution of MFEDs along the length of genome of MNV-2; mean MFED of sequential 99-base fragments are shown.

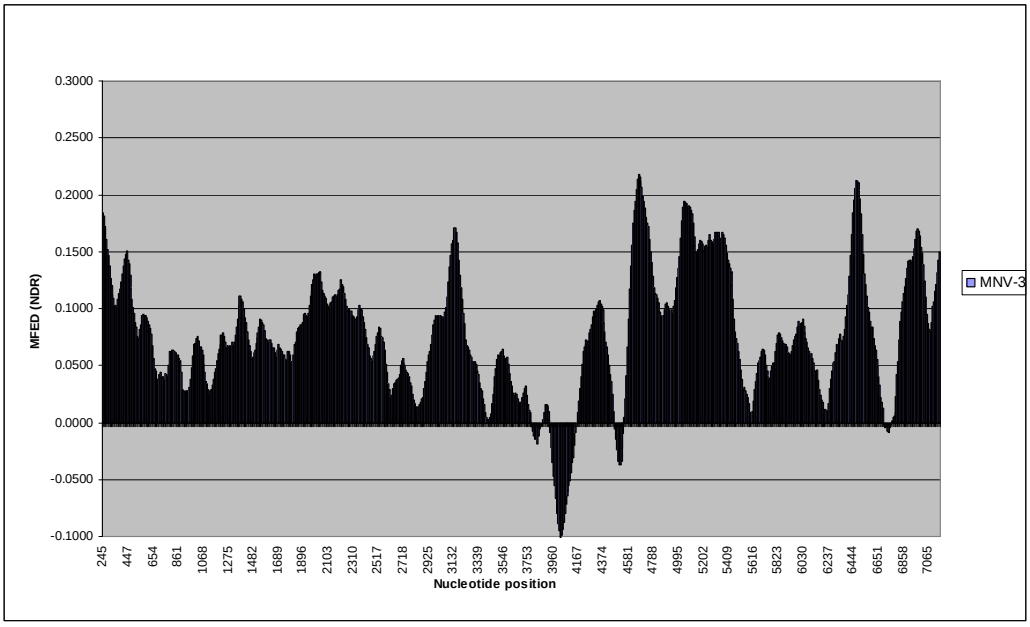


Figure 4.6 C Distribution of MFEDs along the length of genome of MNV-3; mean MFED of sequential 99-base fragments are shown.

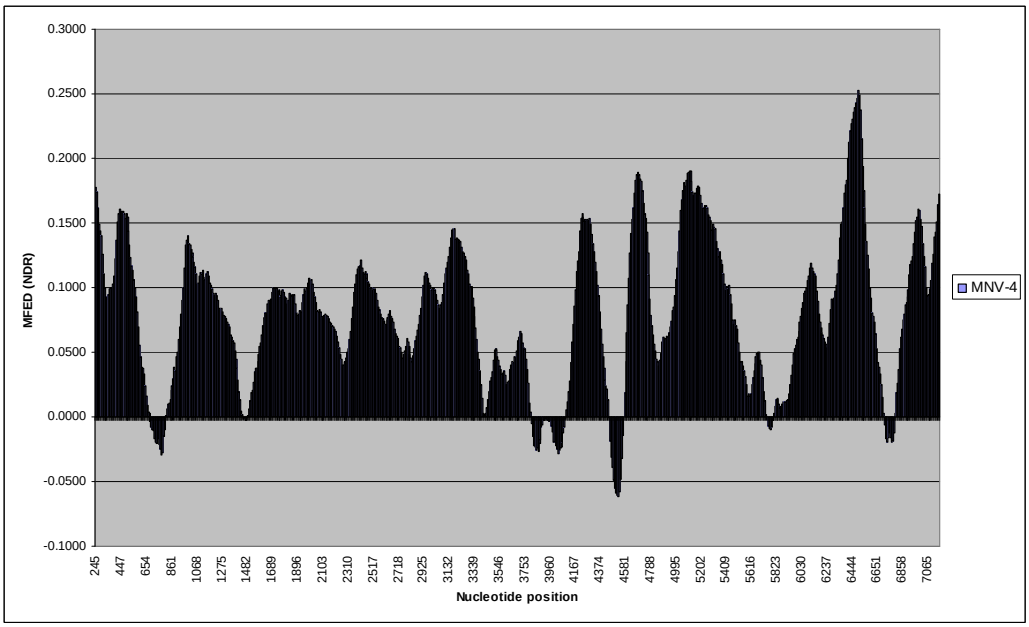


Figure 4.6 D Distribution of MFEDs along the length of genome of MNV-4; mean MFED of sequential 99-base fragments are shown.

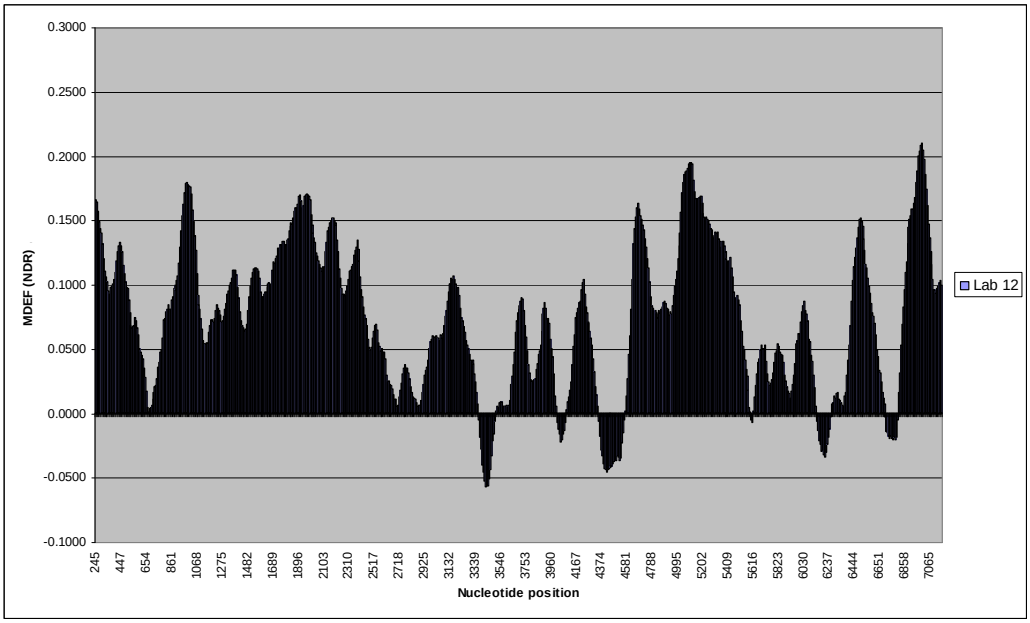


Figure 4.6 E Distribution of MFEDs along the length of genome of MNV from laboratory mouse 12; mean MFED of sequential 99-base fragments are shown.

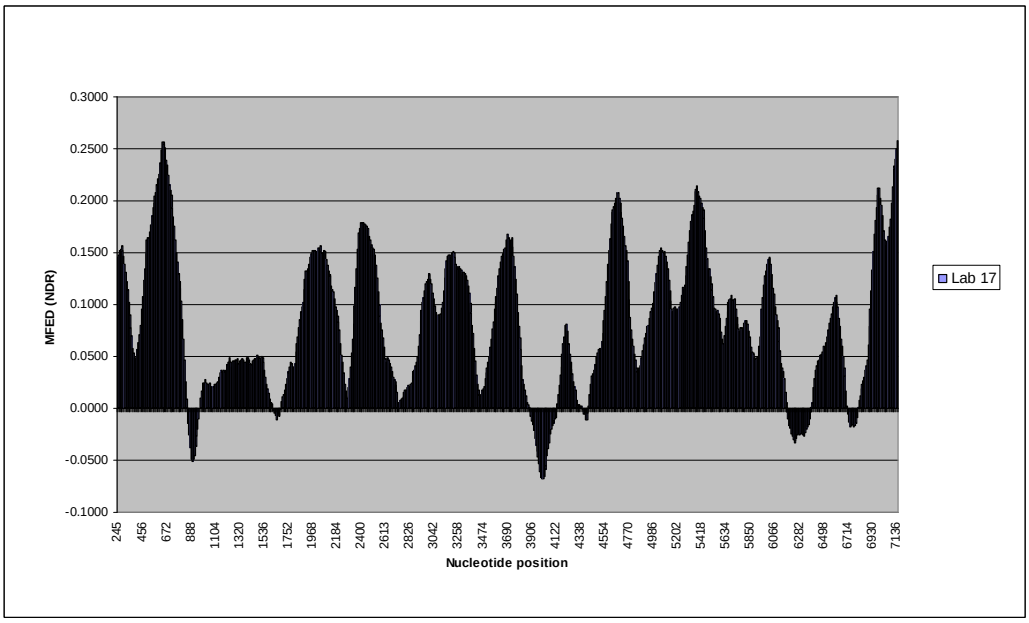


Figure 4.6 F Distribution of MFEDs along the length of genome of MNV from laboratory mouse 17; mean MFED of sequential 99-base fragments are shown.

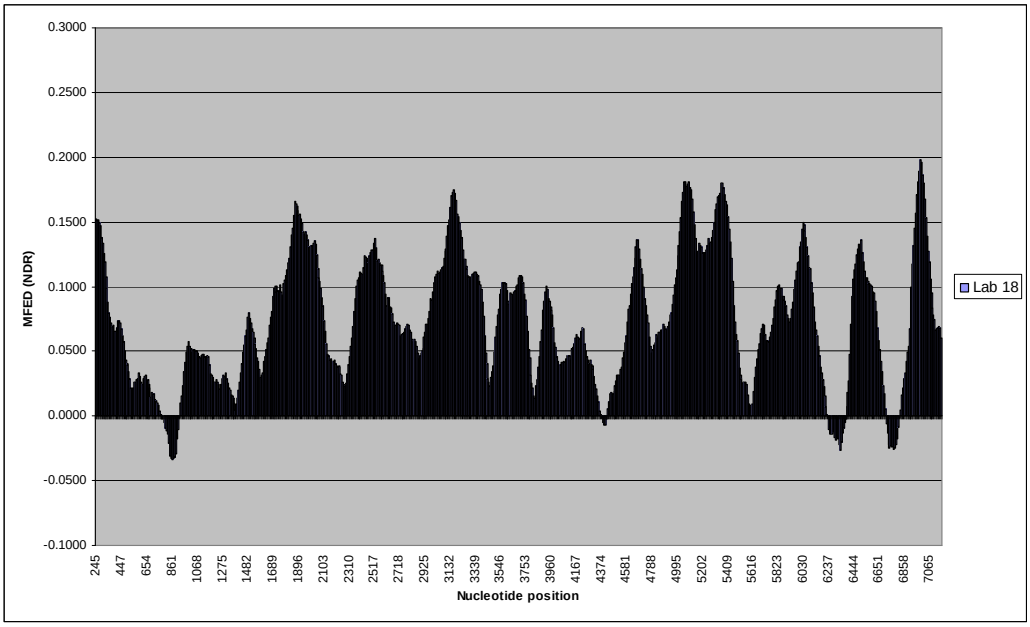


Figure 4.6 G Distribution of MFEDs along the length of genome of MNV from laboratory mouse 18; mean MFED of sequential 99-base fragments are shown.

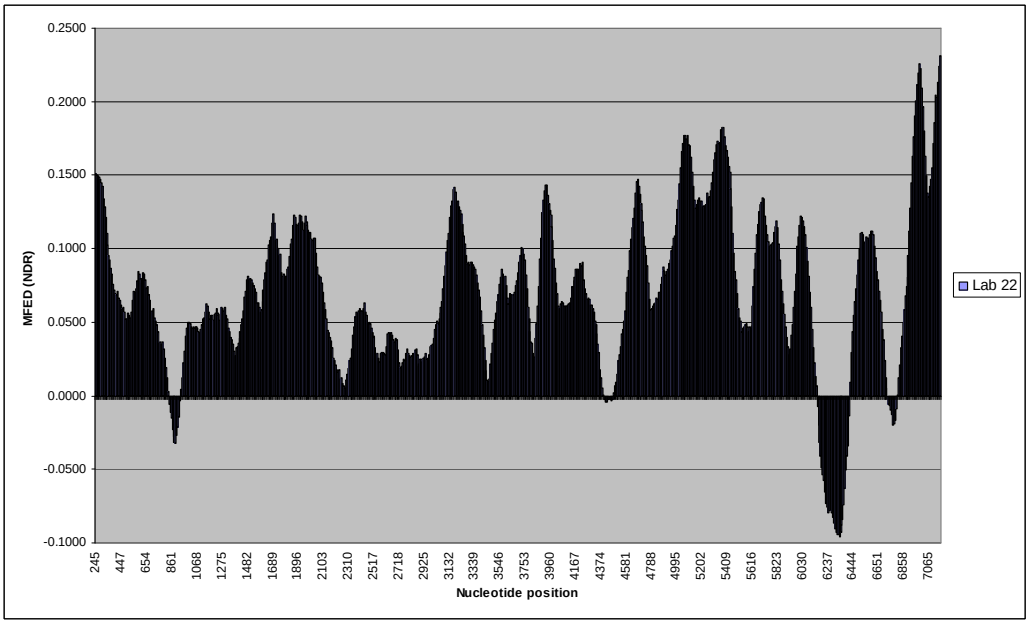


Figure 4.6 H Distribution of MFEDs along the length of genome of MNV from laboratory mouse 22; mean MFED of sequential 99-base fragments are shown.

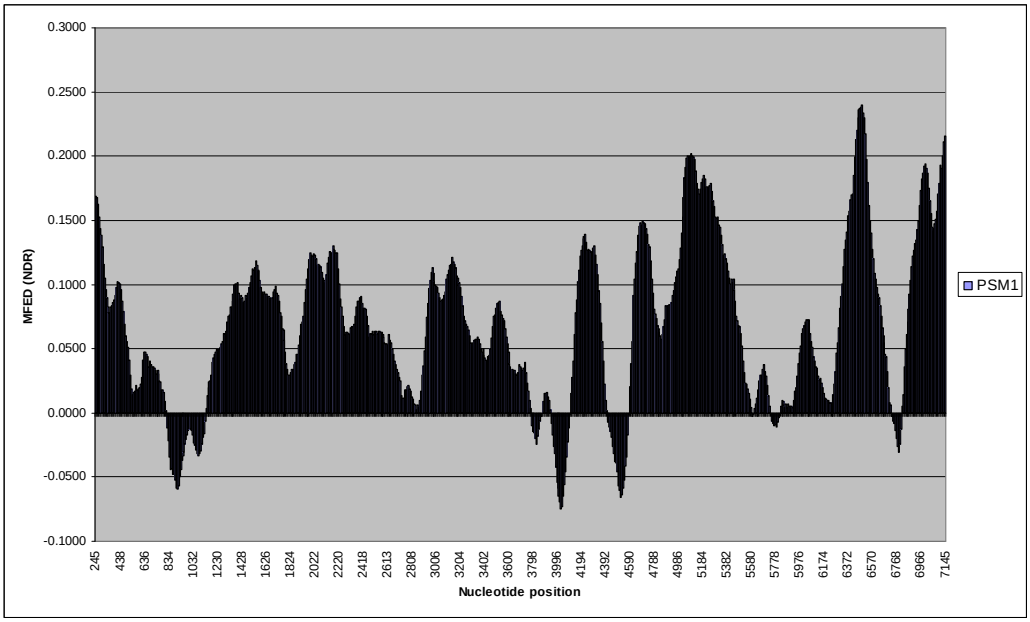


Figure 4.6 I Distribution of MFEDs along the length of genome of MNV from the pet shop mouse, PSM1; mean MFED of sequential 99-base fragments are shown.

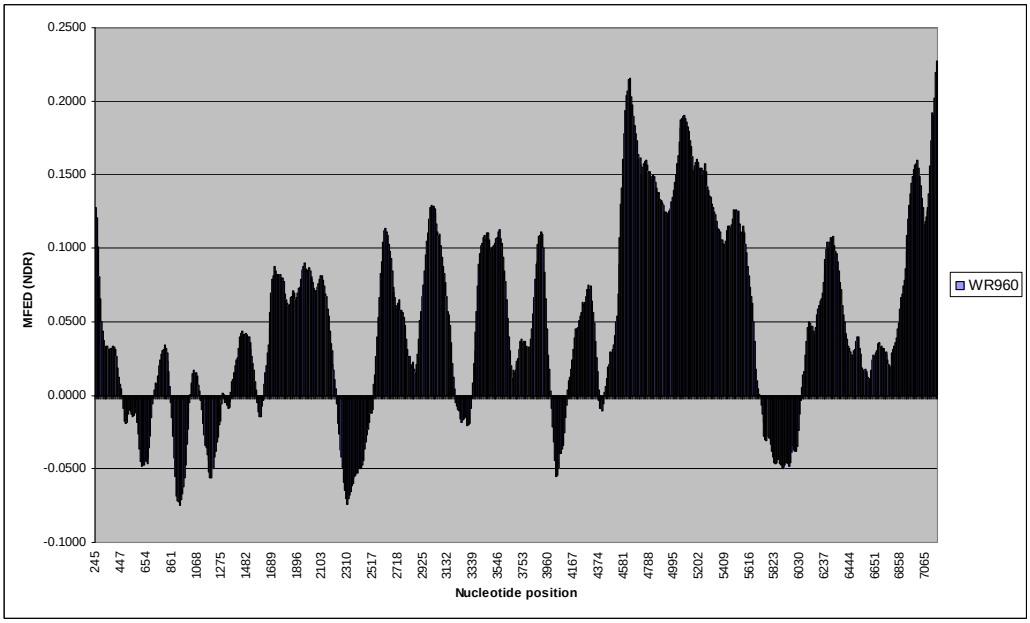
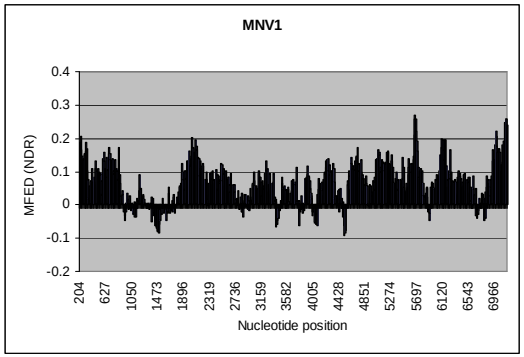
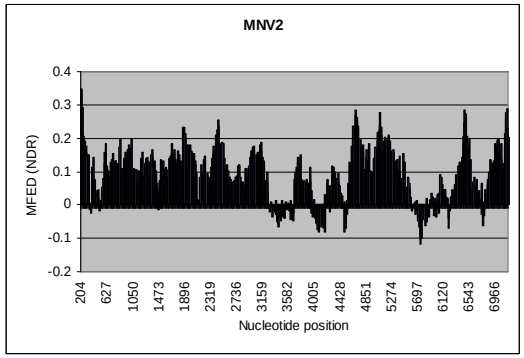
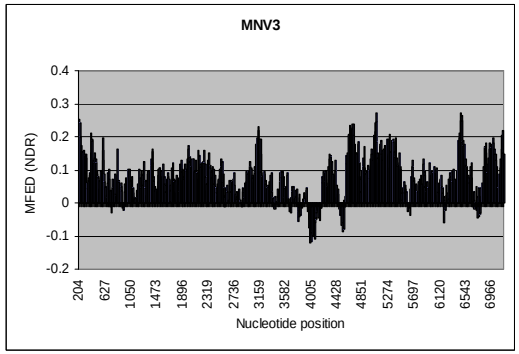
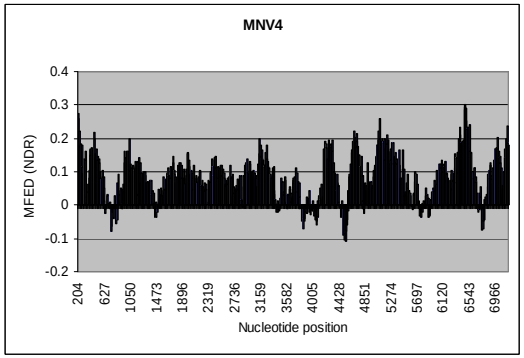


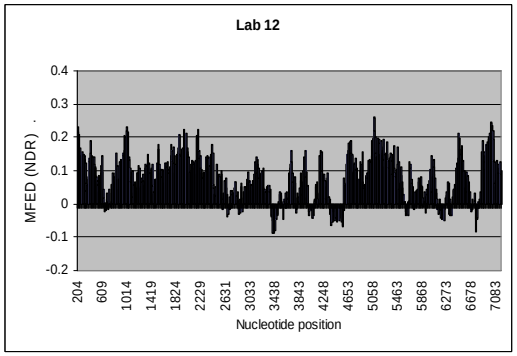
Figure 4.6 J Distribution of MFEDs along the length of genome of MNV from the wood mouse, WR960; mean MFED of sequential 99-base fragments are shown.











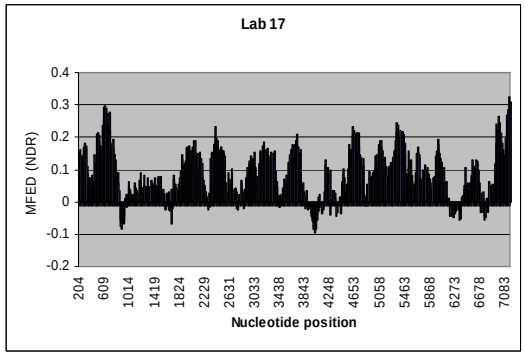
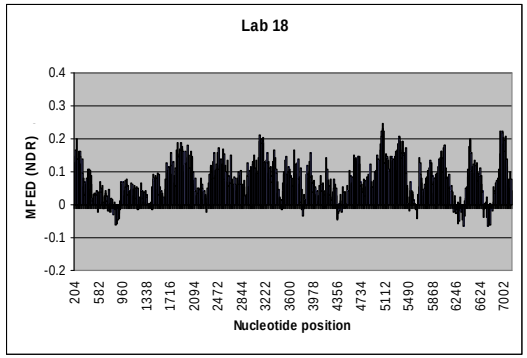
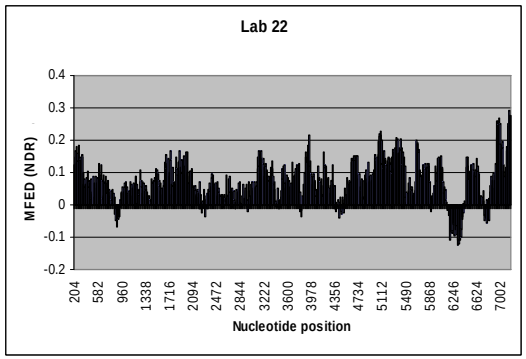
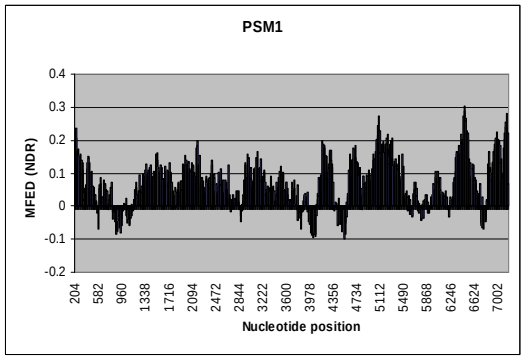


Figure 4.7 Distribution of MFED of all fragments along the genomes of the 10 MNVs as figure 4.6







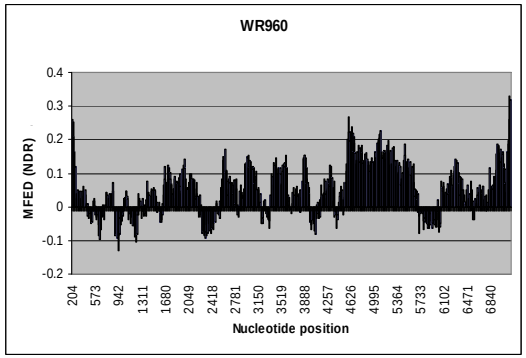


Figure 4.7 cont.

4.3.4 Effect of serial passage

A laboratory strain of MNV-3 was serially passaged, at low and high moi to assess the effect of cell culture adaptation on GORS. At 10 and 20 passages, the viral RNA was extracted and the L1 and L2 regions amplified by PCR and sequenced and compared to the sequence of the original virus. After passage 33, P0 and P33 were sequenced along the length of almost the entire genome and the base mutations recorded. Table 4.4 shows the position of single base mutations identified in MNV-3 after 33 passages, the change in nucleotide and the amino acid.

Position on genome	Nucleotide change	Amino acid change	Change in side-chain properties
608	G-C	Lys-Asn	No change
1200	G-A	Ala-Thr	Non-polar neutral to polar neutral
5960	C-T	Thr-Ile	Polar neutral to non-polar neutral
6152	T-C	Val-Ala	No change
6595	T-A	Phe-Ile	No change
6690	A-G	Thr-Ala	Polar neutral to non-polar neutral
6796	T-G	Phe-Cys	No change

Table 4.4 Showing single base mutations after 33 passages of MNV-3 at high moi, the resulting change in amino acid sequence and the alterations of the amino acid properties

Of these seven point mutations present after passage 33, two are in the non-structural region (608, 1200), four are in the VP1 gene (5960, 6152, 6595) and the other two are in the VP2 gene (6690, 6796). In none of these changes in amino acid sequence is the charged side chain of the amino acids involved altered; and of all these changes only those between alanine-threonine and threonine – isoleucine represent a changes between non-polar neutral and polar neutral. Changing the polarity of the amino acids in the sequence could have an effect on the structure of the protein, and potentially on the antigenicity of the capsid.

4.4 Discussion

MNV has been detected in laboratory mice since its discovery in 2003 (Karst *et al*., 2003). Its prevalence has since been determined in various strains of laboratory mice in research institutions in North America and Germany. In 2005, a study of 12,639 mouse serum samples from laboratory mouse colonies in the United States and Canada revealed a seroprevalence of 22.1 % (Hsu *et al*., 2005). Another study in the United States of sentinel mice in multiple research facilities discovered seroprevalence rates in five facilities of 2 %, 29 %, 49 %, 67 % and 83 %; with 28.5 % cages containing PCR positive faeces (Perdue *et al*., 2007). A 2007 study in Germany of 76 faecal samples collected from two laboratory mouse colonies in Berlin, comprising 28 different mouse lines, showed that MNV was present in 18 mouse lines (64.3 %) and that the prevalence within each mouse line was 83.7 % (Müller *et al*., 2007). We have shown that screening of faecal samples taken from cages of 39 laboratory mouse lines in the University of Edinburgh revealed 26 positive samples, a prevalence of 67 %. This prevalence is comparable to previously published studies. The description of MNV-2, MNV-3 and MNV-4 as persistent viruses i.e. viruses were still detectable in mesenteric lymph node and faeces up to 8 weeks post infection (Hsu *et al*., 2006) and sub-clinical infection may explain the high prevalence rates in laboratory mice. As far as we are aware, this is the first study into the prevalence of MNV in wild mice (*Mus musculus*) as well as three other wild rodent species wood mouse (*Apodemus sylvaticus*), bank vole (*Myodes glareolus*) and field vole (*Microtus agrestis*). The low prevalence of MNV determined in these studies, a single positive result in an *Apodemus* sample, is somewhat surprising as there is a consistently high prevalence in laboratory mice. There may be several explanations for this. The first is that the prevalence is in reality higher but that the PCR is insufficiently sensitive to detect small amounts of virus. In order to detect novel viruses, the primers used were necessarily degenerate to maximise the possibility of finding positive samples. A dilution series of an MNV plasmid was carried out using MNV capsid primers and degenerate norovirus primers. The result is shown in figure 4.8. The MNV capsid primers were able to detect MNV plasmid at a concentration of 5×10^{-7} ng / µl (~4 copies / µl); the degenerate norovirus

primers detected plasmid at 5 x 10^{-6} ng / µl (~40 copies / µl) so were only marginally less sensitive.

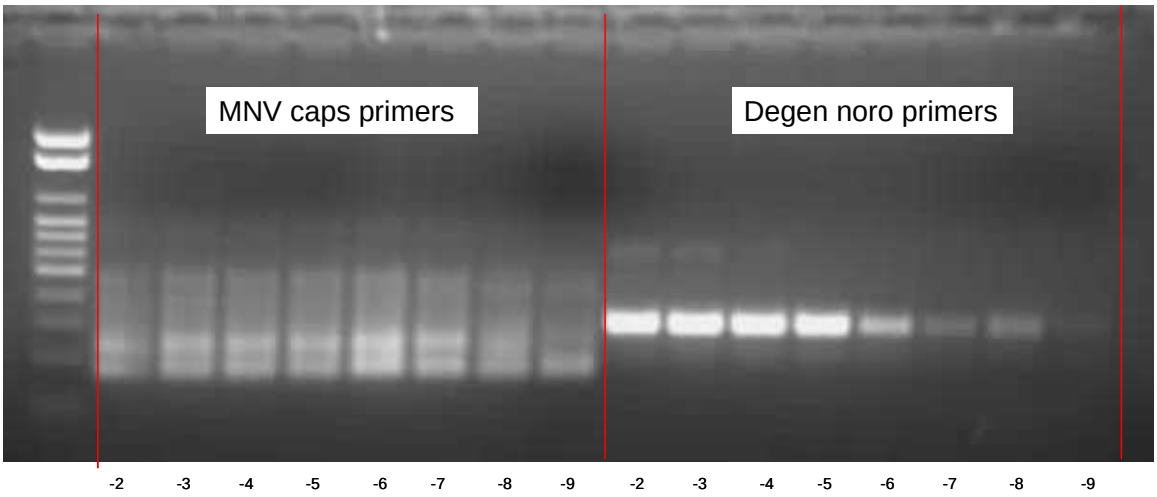


Figure 4.8 PCR of dilution series of pMNV using MNV caps and degenerate norovirus primers; -2 to -9 represent ten-fold dilutions from original plasmid at concentration 500 ng / µl (~4 x 10^{10} copies / µl)

Another factor that may play a part in the discrepancy between the prevalence in laboratory and wild mice is the fact that laboratory strains of mice have deficiencies of the IFN effector molecule Mx (Staeheli *et al*., 1988; Staeheli and Sutcliffe, 1988). Mx1 was first discovered in a strain of mouse unusually resistant to influenza A virus (Lindenmann *et al*., 1962). Subsequent work on Mx1 and Mx2 in human, mouse and rat cells has shown that Mx1 is localised to the nucleus and confers resistance to viruses that replicate in the nucleus; cells expressing rat Mx2 in the cytoplasm show antiviral activity against vesicular stomatitis virus (VSV), and the bunyaviruses Rift Valley fever virus (RVFV) and LaCrosse virus (LACV) (Sandrock *et al*., 2001; Stertz *et al*., 2007). There have been no reports on the relationship between Mx and MNV, but the difference in prevalence between laboratory and wild mice opens a line of further investigation.

A 2006 paper by Zheng and colleagues describes classification and nomenclature of noroviruses. This paper describes the classification of noroviruses into genogroups, clusters (also known as genotypes) and strains based on nucleotide or amino acid sequences. Figures 4.1, 4.3, and 4.4 show phylogenetic trees constructed from nucleotide sequences, whole genome, and

~750 base fragments of the non-structural and structural regions respectively. They show that the MNVs detected in the laboratory faecal samples and the pet shop mice cluster together with the previously published MNV sequences. The sample from the wood mouse though is clearly not as closely associated with the other sequences. These relationships are repeated in trees made from amino acid sequences. Figure 4.2, a tree made from the almost complete amino acid sequence of four novel MNVs discovered in laboratory mice, the pet shop mice and the wild mouse, has an almost identical pattern to the tree made from nucleotides sequences. Figure 4.5 is a phylogenetic tree constructed from the amino acid sequence of the VP1 protein of every genetic cluster/genotype of norovirus throughout the 5 genogroups, including the novel MNVs sequenced in this study. The wood mouse MNV appears to be separate from the other MNVs on the tree, but perhaps just as significantly it does fall within the MNV genogroup, genogroup V, demonstrating that this PCR positive sample from the wood mouse faeces was not a contaminant from a human, porcine or bovine norovirus that the wood mouse had consumed. It is likely, therefore, that this novel MNV was infecting the wood mouse. The classification scheme described in Zheng *et al*., 2006 is based on the amino acid sequence of the major capsid protein VP1, encoded by the sub-genomic ORF2. The most divergent region of the norovirus genome, and consequently the amino acid sequence, is found in the fourth segment of the of P2 domain (Zheng *et al*., 2006). This is to be expected as this is the antigenic region of the capsid and therefore will be under the greatest evolutionary pressure from the interaction of virus and host immunity. The classification scheme described is based on the uncorrected distance method of the complete capsid amino acid sequence as is as follows:

Strain - 0 – 14.1 %

Cluster - 14.3 – 43.8 %

Genogroup- 45.0 – 61.4 %

Table 4.1 shows the p distances all the 6 novel MNVs to have been almost entirely sequenced along all other complete MNV sequences published on GenBank. The status of the MNV from the wood mouse is of most interest as its position on the phylogenetic trees, separate from the other MNVs, raises the possibility that it may be a new member of a new genetic cluster within norovirus genogroup V. In table 4.1, the left-hand most column, in bold,

contains the p distance values of the wood mouse MNV as compared to every other MNV. The values range from 22-23 %, whereas the p distance between all the other MNVs range from 0 – 13 %. Table 4.2 shows the p distances based on the amino acid sequence of the capsid VP1 protein of the 6 novel MNVs identified in this study, along with 12 other previously published MNVs. The wood mouse MNV is again represented in the left-hand column in bold. The p distances between the wood mouse MNV and the others range from 14 -16 %, whereas the values between all the other MNV range from 0 – 7%. According to the classification scheme proposed by Zheng *et al*., this MNV identified in the wood mouse faecal sample may well represent the first member of the new genetic cluster of the MNV genogroup of noroviruses, genogroup V. A much wider study of wild rodents and additional positive samples that could be sequenced and analysed phylogenetically would be necessary to determine if there is indeed a second cluster of MNV present in wild rodents. This discovery certainly makes that likely.

Prediction of secondary structure by an energy minimisation algorithm was carried out on four published MNV sequences, MNV-1, MNV-2, MNV-3 and MNV-4, as well as four newly sequenced MNVs identified by us in laboratory mouse faecal samples, the pet shop mouse faecal sample and wood mouse faecal sample. Figures 4.6 A-J and 4.7 demonstrated the presence of predicted structure throughout the length of the genomes of all the MNV sequences analysed, the defining feature of GORS. The average MFED of all the viruses analysed was above 6 %, a threshold above which viruses may be considered to be a GORS virus, except for the wood mouse MNV, which was just below that value at 5.12 %. The definition of viruses as GORS or non-GORS by average MFED is somewhat arbitrary and the presence of predicted structured regions throughout the length of the genome is probably of greater significance. As structural prediction programs continue to advance it may be possible to more accurately detect specific RNA structures such as stem-loops and pseudoknots more accurately. It is interesting that throughout the genomes are certain areas where there is no predicted structure, but these unstructured regions are not necessarily at the same location of the genome across all the MNVs analysed

Recent work by Davis *et al*., (Davis *et al*., 2008) characterises the physical nature of GORS and supports the thermodynamic predictions. In this study biotin-labelled oligonucleotide probes, designed to hybridise to viral genomic RNA transcripts along the length of the genomes, were used in an accessibility study. This study demonstrated that GORS virus genomes are less accessible to probes, and that non-GORS transcripts are significantly more accessible to the probes. One of the viral RNA transcripts used in that study was MNV; the pattern of probe binding was that the majority of the genome was inaccessible but for occasional areas of accessibility. The secondary structure predictions in this study are consistent with that finding. Davis and colleagues also demonstrated, by inserting the L segment of bunyamwera virus (BV), a non-GORS virus, into HCV, a GORS virus, that the BV segment was still accessible to probes, whereas the flanking HCV genome was still inaccessible to its probes. The interpretation was that GORS consists of a sequence of discrete secondary structures along the length of the genome and is less likely to be a global RNA structure dependant on tertiary structure. The predicted structured regions along the MNV genomes in this study are therefore probably similar in nature, and are likely to be a series of discrete secondary structure motifs such as stem-loops. The presence of extensive secondary structure in the wood mouse MNV is also consistent the observed distribution of GORS. GORS appears from all studies so far to be conserved within genera or virus groups, although virus families may contain GORS and non-GORS genera. Within the family *Caliciviridae*, the genus *Norovirus* contains five distinct genogroups. Human noroviruses are predicted to be non-GORS viruses and infection causes an acute and quickly resolved illness. MNV is a GORS virus which is persistent in its natural host. The classification of the wood mouse norovirus as an MNV, meant that its classification also as a GORS virus was expected.

The association of GORS with persistence led to the hypothesis that it confers an evolutionary advantage to those viruses that are GORS by, in some way, protecting the virus from being cleared by the host's immune response. As viruses undergo adaptations in cell culture the passage study was designed to assess the impact of multiple passage on the presence of GORS in MNV. The result was that after 33 passages at high moi there were only seven base

mutations, a mutation rate of 0.1 %. This was not considered significant enough to warrant bioinformatics analysis as it was considered unlikely that this degree of sequence drift would alter the secondary structure predictions. The hypothesis was that in cell culture, with no pressure from a fully intact immune system in an animal, that the advantage that viruses gain from maintaining extensive secondary structure in their RNA genomes would be lost, and that a GORS virus would lose its widespread structure. One interpretation of the result of this study is that the RAW 264.7 cells, in which MNV is propagated, do not constitute an environment in which the pressure to maintain GORS has been lost. The base mutations that were observed lead to non-synonymous changes in the amino acid sequence, and three of those changes three were changes that included an alteration in polarity if the side chain. RNA viruses replicate their genomes using RNA dependant RNA polymerases which have no proof-reading capacity, so throughout the course of multiple passage it is likely that many mutations occur that are detrimental to the virus, so are never passed on. The changes observed have been selected by the conditions present in the cell culture system. The nature of the evolutionary pressure operating on viruses in cell culture is different from those found in animals. In order to investigate this further it would be necessary to repeat this study in cell lines in which various parts of the innate immune system had been knocked-out or knocked-down or otherwise inhibited. The relationship between GORS and innate immunity will be discussed later, but a recent study has shown that MNV-1 is detected in dendritic cells by MDA-5 (McCartney *et al*., 2008). It is not known if the same is true of MNV infection of macrophages, the primary cell type that MNV replicates in, although dendritic cells are related to macrophages. A study in which the various parts of the MDA-5 cascade in IFN-β induction were knocked out of RAW 264.7 cells, a murine macrophage cell line, and the effect on mutation rate and degree of secondary structure in MNV may help to shed light on the biological relationship between the presence of GORS in viral genomes and the ability of GORS viruses to persist in their natural hosts.

Chapter 5 A Study of the Interactions of GORS and non-GORS RNA Transcripts With Cellular RNA Stability Mechanisms and Innate Cellular Immunity

Chapter 5 A Study of the Interactions of GORS and non-GORS RNA Transcripts With Cellular RNA Stability Mechanisms and Innate Cellular Immunity

5.1 Introduction

The observation that viruses that have GORS are able to establish persistent infections led to speculation about possible mechanisms by which the presence of extensive secondary structure throughout the RNA genome might lead to persistence. In the original study describing the discovery of GORS (Simmonds *et al*, 2004) potential mechanisms are discussed in relation to the interactions between RNA and the cellular innate immune machinery. Eukaryotic cells and viruses have been co-evolving throughout the history of life on earth and there are many mechanisms that cells have evolved to counter viruses, and viruses have evolved as many means of avoiding those mechanisms.

Plants and many invertebrates use RNA interference (RNAi) as an innate antiviral mechanism. Infection by RNA viruses results in the production of long dsRNAs, e.g. the replicatory intermediate. The RNAi mechanism first described by Napoli *et al*. in 1990(reviewed by Umbach and Cullen, 2009) consist of these long dsRNAs being cleaved by Dicer resulting in a pool of small, ~22 base pair, dsRNAs. One strand of these RNAs, called siRNA duplexes, is loaded onto the RNA-induced silencing complex (RISC). RISC then binds via the RNA to complementary mRNA, viral and/or cellular, resulting in degradation. Plant and invertebrate viruses have evolved mechanisms to counter the RNAi system e.g. tomato bushy stunt virus p19 protein binds 21 base pair dsRNA to prevent its being associated with RISC (Scholthof, 2006).

The most important innate immune system in mammalian cells is the IFN response and whether RNAi is used as an antiviral mechanism is unclear. dsRNA produced by viruses during infection is the most important pathogen associated molecular pattern (PAMP) to induce IFN (Marcus, 1983). The pattern recognition receptors (PRRs) responsible for detecting dsRNA are TLR3, which detects intraendosomal dsRNA (Alexopoulou *et al*., 2001); and the cytoplasmic receptors RIG-I and MDA-5 (Yoneyama *et al*., 2004; Andrejeva *et*

al., 2004). These receptors feed into two transductions cascades that act through NF-κB and IRF-3 which act on the IFN-β promoter resulting in the expression of IFN-β (as discussed earlier and reviewed by Randall and Goodbourn, 2008). Of the IFN induced antiviral effectors, PKR and 2'5'-OAS are also stimulated by dsRNA. Stimulation of 2'5'-OAS leads to the activation of the endoribonuclease RNase L (Zhou *et al*., 1993). RNase L has a role in antiviral immunity by cleaving viral and cellular RNAs, creating short RNAs. It has been reported that these short RNAs, especially of cellular origin, are then detected by RIG-I or MDA-5 leading to further IFN induction (Malathi *et al*., 2007).

The mechanisms by which the GORS leads to persistence have been speculated to be to shield the viral RNA in some way from the cell's PPRs. Many viruses are able to establish persistent infections by evasion of innate and/or adaptive immunity by producing proteins that inhibit various parts of the IFN response; from inhibition of IFN induction to inhibition of IFN stimulated enzymes. Evasion of immunity does not necessarily lead to persistence though, as many non-persistent viruses have evolved strategies to avoid the effects of the various antiviral cellular mechanisms. dsRNA can be degraded or sequestered during several viral infections (see table 1.2), reducing the degree of IFN induction. We speculated that the evolution of GORS in those virus genera that have it, gives those viruses an advantage as this feature of their RNA genomes would enable them to evade detection, perhaps by mimicking cellular RNAs that also have structured elements. We hypothesised that upon viral infection of a cell and uncoating of the genome, genomic RNA from GORS viruses would induce a weaker IFN response than genomic the RNA from non-GORS viruses. The main challenge in testing this hypothesis is that GORS is a phenomenon that exists in some genera of positive sense RNA virus families, but not others, across several families. In order to investigate a biological role of GORS we needed to select a panel of RNA viruses that were predicted to be GORS and non-GORS. Studying active infections by viruses from different families, which may have different replication strategies and grow in different cell lines, makes a direct comparison between a GORS group and non-GORS group difficult. In order to study the effect of GORS alone, RNA transcripts were made of the genomes of a panel of predicted GORS and non-GORS viruses. These

transcripts, which were replication incapable, were then used in studies in various cell lines: the mouse fibroblast, NIH3T3; and the human type II pulmonary epithelial cell, A549. In this way the effect of all the viral RNA transcripts on the cell e.g. IFN induction, can be measured simultaneously under the same conditions. The effect on the RNA of being introduced into the cellular environment could also be studied. Using replication incapable RNAs removes potentially complicating factors such as RNA replication and translation of protein during virus infections.

The following viral RNA transcripts were made by Dr Matthew Davis from infectious clones kindly provided by others (see material and methods for acknowledgements).

GORS	**Genus**	**Family**
GBVirus-C (GBV-C)	GBV-C like	*Flaviviridae*
Hepatitis C virus (HCV)	*Hepacivirus*	*Flaviviridae*
Murine norovirus (MNV)	*Norovirus*	*Caliciviridae*
Pea enation mosaic virus 2 (PEMV2)	*Umbravirus*	
Non-GORS		
Bunyamwera virus (BV)	*Orthobunyavirus*	*Bunyaviridae*
Poliovirus (PV)	*Enterovirus*	*Picornaviridae*
Rubella virus (RV)	*Rubivirus*	*Togaviridae*
Semliki Forest virus (SFV)	*Alphavirus*	*Togaviridae*
Theilers murine encephalitis virus (TMEV)	*Cardiovirus*	*Picornaviridae*

Table 5.1 Table of viruses used in this study

Short notes on virus entry and uncoating, and genome structure of those viruses not previously discussed will be given here, as these are the events that would lead to the potential exposure of genomic viral RNA to the cellular PRRs during infection. The viruses of the family *Flaviviridae* are enveloped, positive-sense, singe-stranded RNA viruses. HCV is classified in the genus *Hepacivirus*

whereas GBV-C is classified in an unnamed genus within the family. HCV enters cells, predominantly hepatocytes, and is internalised through clathrin-mediated endocytosis; the genome is then uncoated although the mechanism is still unclear; replication then occurs in a membrane-bound replication complex (Moradpour *et al*., 2007; Brass *et al*., 2009). GBV-C has a tropism for human lymphoid cells and although little is known about its replication it is likely to be similar to that of other flaviviruses. The genomes of HCV and GBV-C are similar though, being ~9.6 kilobases in HCV and 9.1-9.3 kilobases in GBV-C. The 5' ends of the genomes consist of highly conserved UTRs that fold into complex secondary structures consisting of stem-loops and pseudoknots that act as an IRES. The genomes are arranged with the structural genes at the 5' end and the non-structural proteins at the 3' end. The last non-structural protein adjacent to the 3' UTR, NS5B, is the RNA dependant RNA polymerase. HCV genome replication occurs associated with the plasma membrane of the endoplasmic reticulum (Lindenbach and Rice, 2001, Fundamental Virology ed. Knipe and Howley; Reshetnyak *et al*., 2008; Nakamura *et al*., 2008).

The family *Togaviridae* consist of two genera, *Alphavirus* and *Rubivirus*; they are enveloped, positive-sense, single-stranded RNA viruses. The alphavirus genome is ~11 kilobases whereas rubella virus (the only member of the genus *Rubivirus*) has a genome of 9.8 kilobases. Their genomes are arranged with the non-structural genes occupying the 5' two thirds of the genome and the structural genes the 3' third. The structural proteins are translated from a sub-genomic RNA. The 5' end of the genome is linked to a 7-methylguanosine cap and the 3' end has a poly-A tail (Kuhn, Fields Virology 5th Edition, Ed Knipe and Howley, 2007). Four conserved regions of the genome have been identified as cis-acting replication elements (Ou *et al*.,1981; 1982; 1983). Semliki Forest virus is a member of the *Alphavirus* genus. Alphaviruses have a broad host range (reviewed by Strauss *et al*., 1994), due to either the presence of binding sites to multiple receptors or the presence of a ubiquitous receptor in many cell types across a broad range of species. Alphaviruses enter cells via an endocytic pathway (Marsh *et al*., 1983), the endosome is acidified causing fusion between viral and cellular membranes resulting in the release of the genomic RNA into the cytoplasm. The protein NS1 has an affinity for the lipids found in plasma

membranes leading to the location of replication complexes associated with cytoplasmic vacuoles (Kujala *et al*. 2001). Rubella virus is also thought to enter cells via receptor-mediated endocytosis followed by acidification and release of the genome (Katow and Sugiura, 1988).

The genomes of viruses of the family *Bunyaviridae* consist of three segments of single-stranded, negative or ambi-sense RNA. The three segments are designated small (S), medium (M) and large (L). Bunyaviruses enter the cell via receptor-mediated endocytosis. Acidification of the endosome leads fusion of viral and cellular membranes resulting in the release of genomic RNA into the cytoplasm. The negative sense RNAs are transcribed into mRNA and the replication complexes are formed in tubular structures made from Golgi elements (Fontana *et al*., 2008). The principle site of replication of orthobunyaviruses within the body is the skeletal myocyte, but viraemic spread results in the viruses entering the central nervous system where they replicate in neurones and glial cells (Schmaljohn and Nichol, Fields Virology 5th Edition, Ed Knipe and Howley, 2007).

Pea enation mosaic virus 2 (PEMV-2) is a member of the genus *Umbravirus*, its family classification is uncertain. Umbraviruses are plant viruses; they have a single-stranded, positive sense RNA genome, they are rather short genomes, PEMV-2 has a genome length of 4253 nucleotides. The genome consists of three ORFs but there is little information on the mechanisms of replication (Taliansky and Robinson, 2003).

Details of the genomes and replication strategies of caliciviruses and picornaviruses have already been given in chapters 1 and 3 respectively. It is notable that all of the viruses discussed share similar strategies in the early stages of their life cycles. After viral entry there is an uncoating event which allows the viral RNAs to be translated. The majority of the viral replication then takes place within replication complexes that incorporate organelle membranes, lipids and viral proteins. The studies presented in this chapter involved the electroporation or transfection of RNA transcripts into the cytoplasm of cells in

order to mimic the presence of genomic viral RNA after it's uncoating and exposure.

The RNA used in these studies were made replication incapable either by cutting of the plasmid with restriction enzymes to remove part of the genome (the picornaviruses and flaviviruses were truncated at their 3' ends to obliterate their RNA dependant RNA polymerase genes); by use of plasmids that contained only part of the genome (the SFV clone contained only the non-structural genes); or in the case of bunyamwera virus, only using one of the three genomic RNA strands. Table 5.2 shows the restriction enzymes used and the points of truncation.

Virus	Restriction enzyme	Truncation point	Nucleotides lost	Total length
GBV-C	MluI	8862/9395	533	8862
HCV	EcoRV	9236/9678	442	9236
PV	PvuII	7055/7440	385	7055
TMEV	RsrII	6724/8101	1377	6724
RV	BamHI	9174/9762	588	9174
BV	N/A	N/A	N/A	6875
MNV	N/A	N/A	N/A	7400
SFV	N/A	N/A	N/A	7400
PEMV2	N/A	N/A	N/A	4200

Table 5.2 Table showing truncated viruses, the restriction enzymes used, point of truncation and number of nucleotides lost and total transcript length

5.2 Objectives

1. Study of RNA stability in GORS and non-GORS viruses

The rate of RNA creation and degradation is one of the most important means of regulation of gene expression in the cell (reviewed by Garneau *et al*., 2007). Cellular mRNAs have 5' 7-methylguanosine caps and 3' poly(A) tails, and their degradation is normally carried out by exonucleases from either end. We hypothesised that GORS viruses would be less susceptible to ribonuclease degradation, allowing the genomes to exist for longer in the cytoplasm so that replication could be continuous in a persistent infection. In this study, 500 ng of the full length or truncated transcripts of 3 GORS viruses and 3 non-GORS viruses were electroporated into NIH3T3 cells and their degradation measured over 72 hours by quantitative PCR.

2. Study of IFN-β induction in GORS and non-GORS viruses

The hypothesis is that GORS viral RNA transcripts induce IFN-β less strongly than non-GORS viral RNA transcripts. To test this hypothesis the full length/truncated transcripts were transfected into cells and the level of IFN-β induction measured 18 hours post-transfection by two methods: quantitative real-time PCR and by a luciferase reporter assay. All studies using the luciferase reporter system were conducted in collaboration with Dr Selena Sagan.

5.3 Results

5.3.1 Stability of GORS and non-GORS RNA transcripts in NIH3T3 cells

Full length or truncated RNA transcripts were made by *in vitro* transcription from infectious clones as shown in table 5.2 from three viruses predicted to be GORS viruses and three non-GORS. The predicted GORS viruses were: GBV-C, HCV and MNV; the non-GORS viruses were BV, PV and TMEV. In order to measure the degradation of RNA it was necessary to create a standard curve for use in the real-time PCR. Series of ten-fold dilutions were made of each RNA transcript before undergoing reverse transcription under the same conditions. These series of cDNA were then used as standard curves during the real-time PCR of each virus, which are presented in figure 5.1 along with melt curve analysis deomstrating each PCR produced a single specific product at a satisfactory reaction efficiency. The full length or truncated transcripts were made as described from infectious clones of each virus by *in vitro* transcription; their concentrations were measured by spectroscopy and their concentration normalised to 100 ng / µl. The RNA transcripts underwent overnight treatment with Turbo DNAse (Megascript kit, Ambion) and the restriction enzyme HaeIII (New England Biolabs) to remove residual DNA, ensuring the PCR would detect only the RNA electroporated into the cell during the experiment. A 12-well plate was seeded with 1 x 10^5 cells 24 hours pre-electroporation. The time of the electroporation of 500 ng of each transcript into NIH3T3 cells was considered 0 hours, the mass of the RNA transcripts was measured at 4, 24, 48 and 72 hours. The degradation of the transcripts was recorded as percentage decline from 4 hours on a logarithmic scale, which is shown in figure 5.2. The transcripts were stable for the first 24 hours; they then declined at a similar rate over the next 48 hours. Although the six viruses do not degrade identically, there is no difference in the rate of degradation between GORS and non-GORS viruses and the lines on the graph follow a similar course.

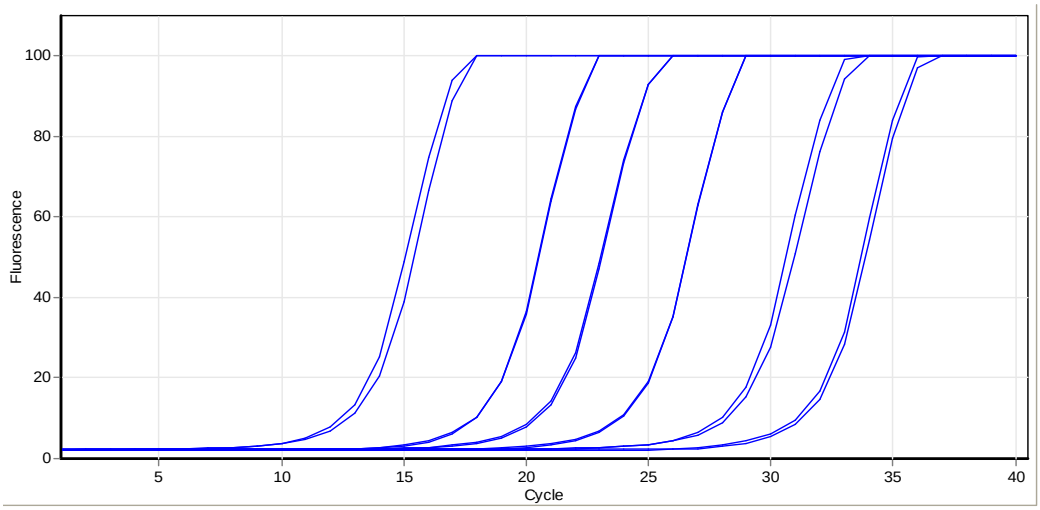


Figure 5.1 A(a)) Standard curve of GBV-C cDNA; made from serial dilutions of RNA, from 1 ng/µl – 1 x 10^{-5} ng/µl

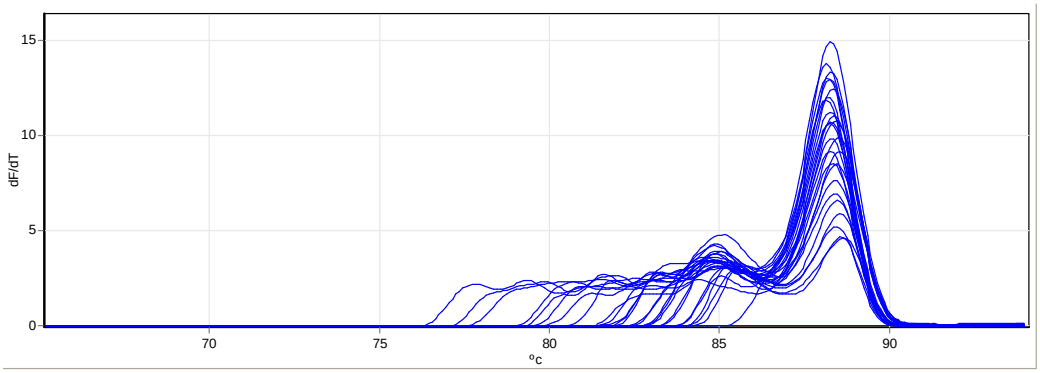


Figure 5.1 A(b) Melt curve analysis of GBV-C qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	0.80667 (* = 10^(-1/m) - 1)
M	-3.89287
B	0.35811
R Value	0.99598
R^2 Value	0.99198

Figure 5.1 A(c) Efficiency data of GBV-C qPCR

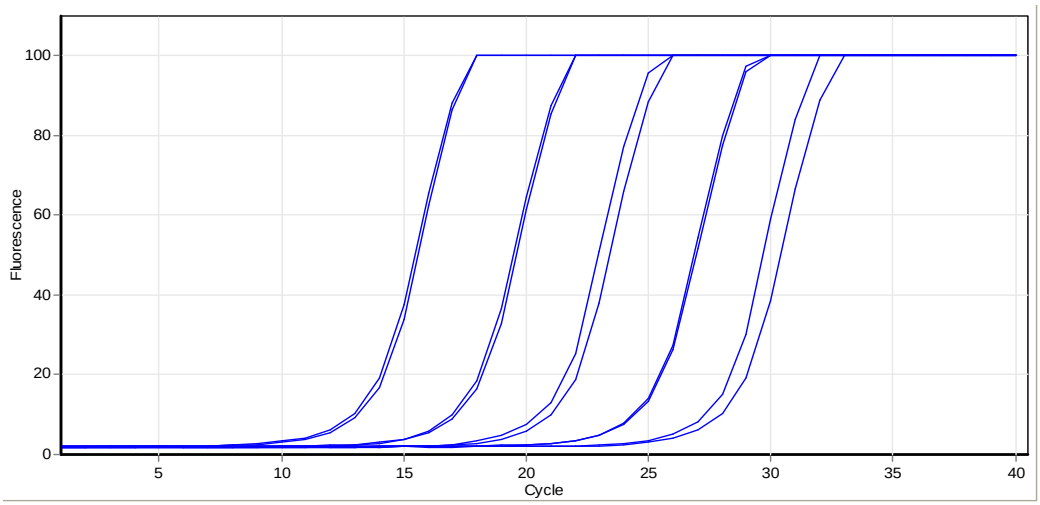


Figure 5.1 B (a) Standard curve of HCV cDNA; made from serial dilutions of RNA, from 1 ng/µl – 1 x 10^{-4} ng/µl

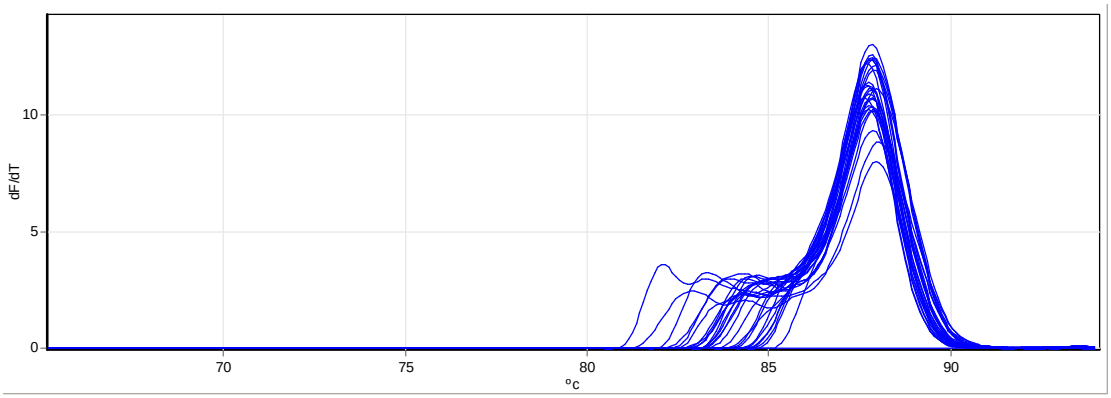


Figure 5.1 B(b) Melt curve analysis of HCV qPCR; single prominent peak indicates a single specific PCR product

efficiency (*)	1.5173 (* = 10^(-1/m) - 1)
M	-2.49417
B	1.75876
R Value	0.96397
R^2 Value	0.92923

Figure 5.1 B(c) Efficiency data of HCV qPCR

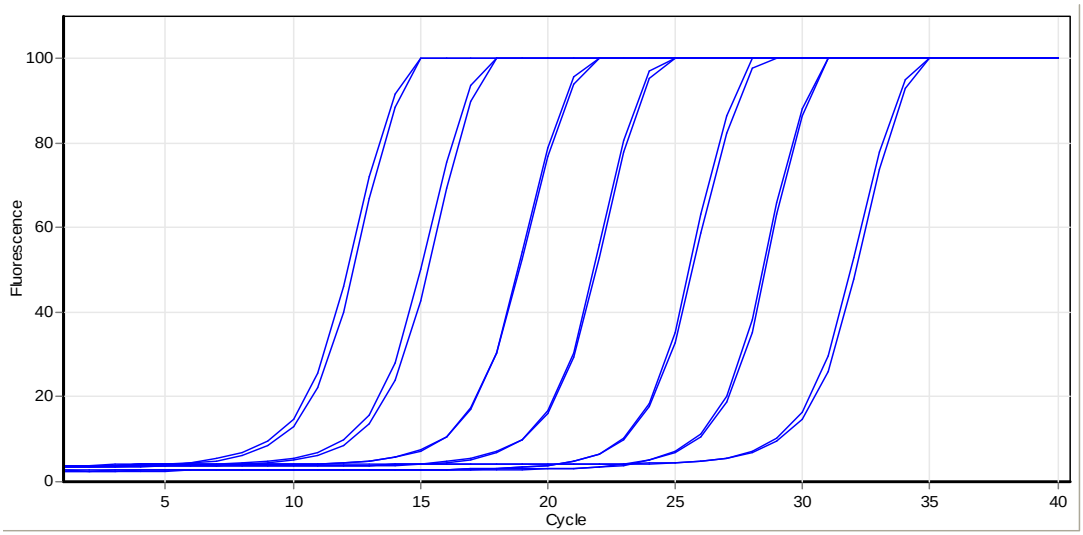


Figure 5.1C (a) Standard curve of MNV cDNA; made from serial dilutions of RNA, from 1 ng/µl – 1 x 10^{-6} ng/µl

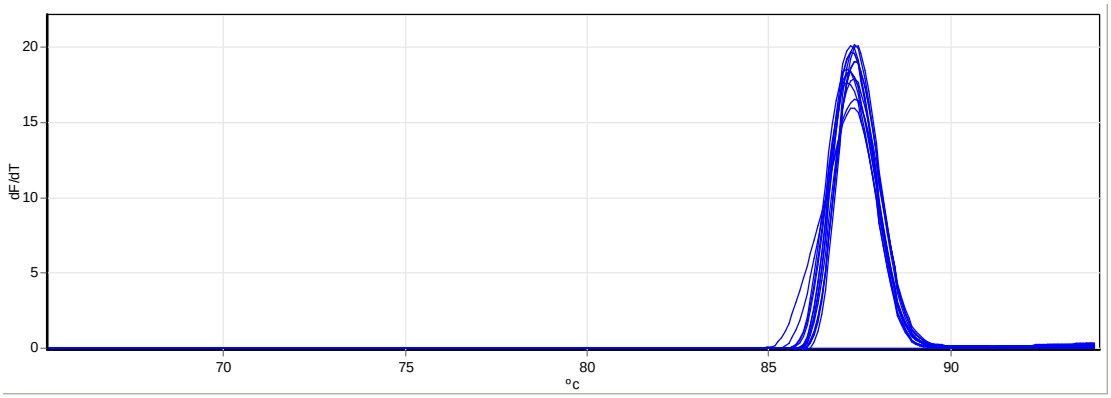


Figure 5.1 C(b) Melt curve analysis of MNV qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	1.02593 (* = 10^(-1/m) - 1)
M	-3.26132
B	-2.4031
R Value	0.99928
R^2 Value	0.99857

Figure 5.1 C(c) Efficiency data of MNV qPCR

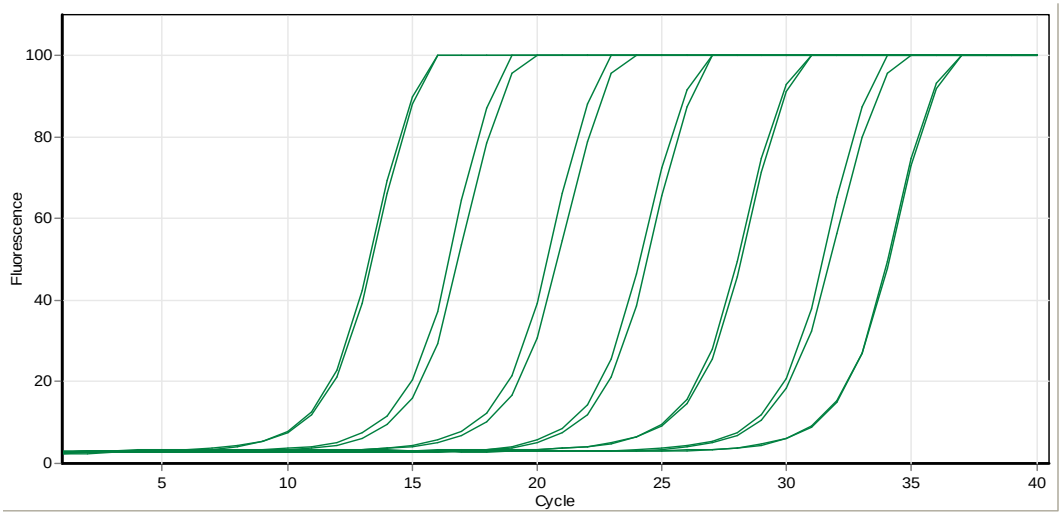


Figure 5.1 D(a) Standard curve of BV cDNA; made from serial dilutions of RNA, from 1 ng/µl – 1 x 10^{-6} ng/µl

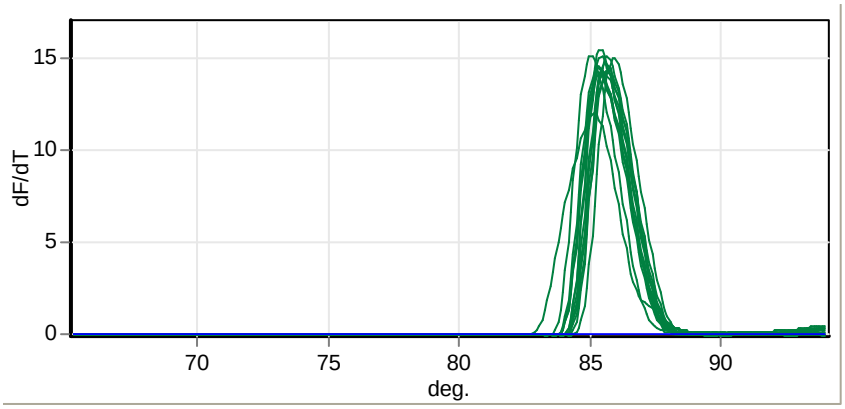


Figure 5.1 D(b) Melt curve analysis of BV qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	0.9189 (* = 10^(-1/m) - 1)
M	-3.53291
B	-0.76528
R Value	0.99843
R^2 Value	0.99686

Figure 5.1 D(c) Efficiency data of BV qPCR

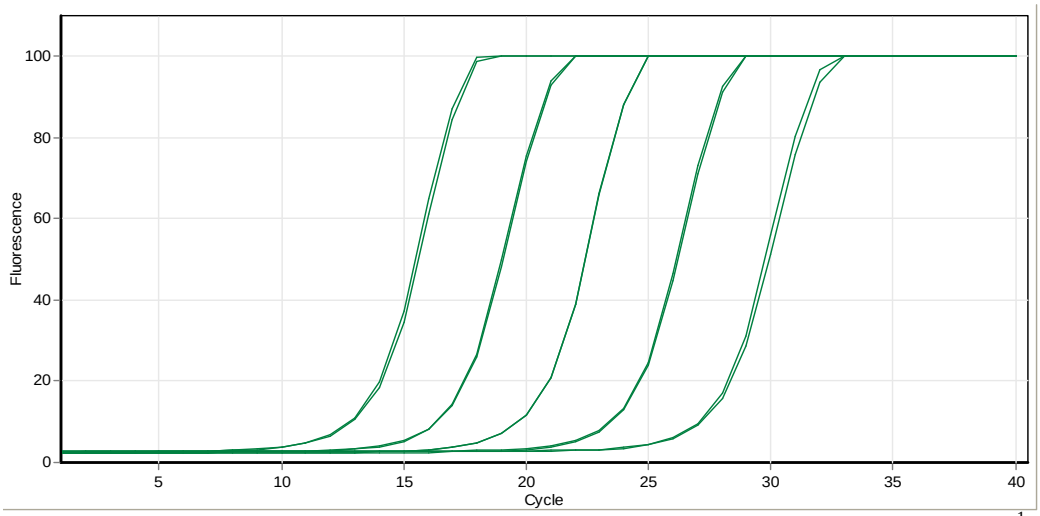


Figure 5.1E (a) Standard curve of PV cDNA; made from serial dilutions of RNA, from 1 x 10^{-1} ng/µl - 1 x 10^{-5} ng/µl

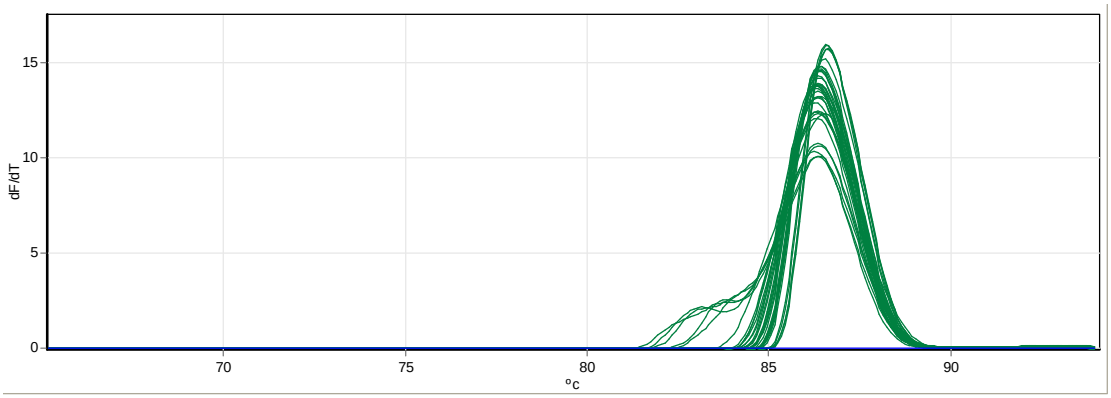


Figure 5.1 E(b) Melt curve analysis of PV qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	0.95017 (* = 10^(-1/m) - 1)
M	-3.4474
B	-6.02105
R Value	0.99964
R^2 Value	0.99928

Figure 5.1 E(c) Efficiency data of PV qPCR

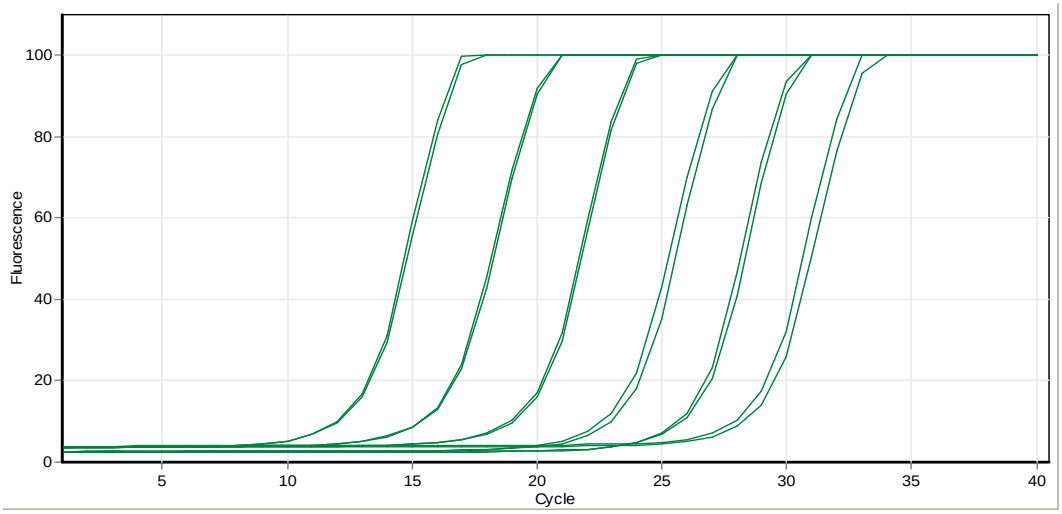


Figure 5.1F (a) Standard curve of TMEV cDNA; made from serial dilutions of RNA, from 1 x 10^{-1} ng/µl - 1 x 10^{-6} ng/µl

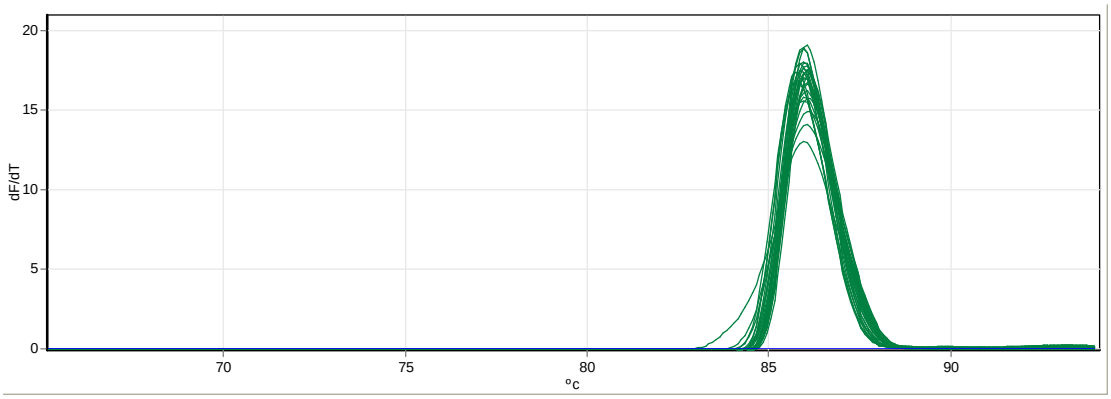


Figure 5.1 F(b) Melt curve analysis of TMEV qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	1.11492 (* = 10^(-1/m) - 1)
M	-3.07414
B	0.12921
R Value	0.99842
R^2 Value	0.99684

Figure 5.1 F(c) Efficiency data of TMEV qPCR

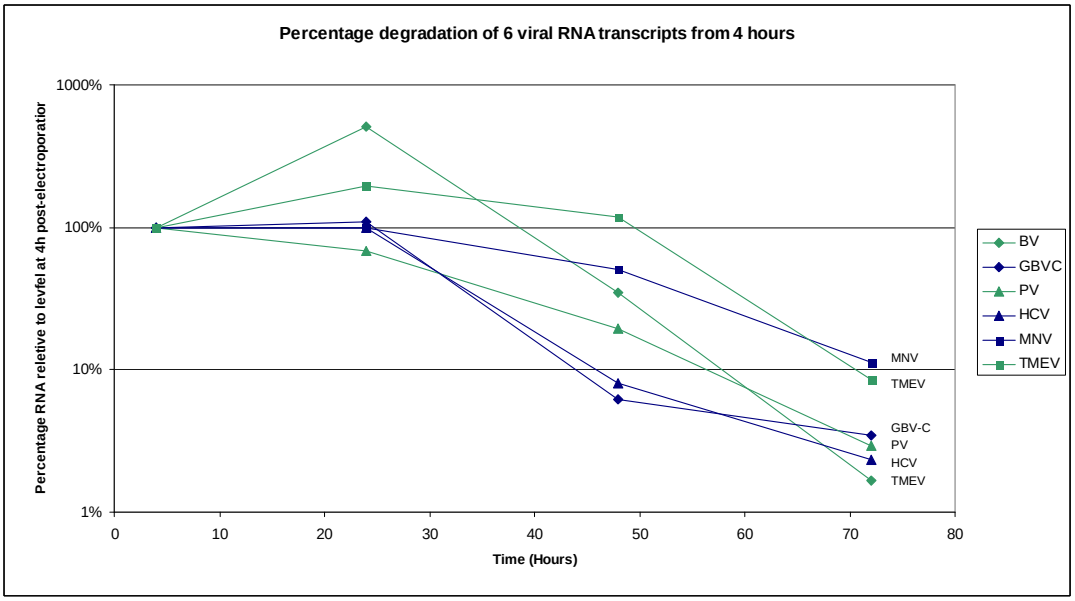


Figure 5.2 Graph showing % decline of 6 viral RNA transcripts; GBV-C, HCV and MNV (blue points) are GORS viruses; BV, PV and TMEV (green points) non-GORS viruses; each sample was tested in duplicate. There is no difference between the rate of degradation between GORS and non-GORS.

	4 h	24 h	48 h	72 h
BV	33.00%	298.85%	4.99%	0.67%
GBVC	32.37%	18.83%	1.03%	1.19%
PV	21.58%	8.37%	5.48%	1.36%
HCV	69.12%	72.35%	1.92%	0.57%
MNV	14.06%	10.90%	7.99%	2.30%
TMEV	21.34%	57.38%	76.45%	2.58%

Table 5.3 Standard deviations of % changes at each time point for each virus

5.3.2 IFN-β induction by full length/truncated RNA transcripts of GORS and non-GORS viruses in NIH3T3 cells and A549 cells

IFN-β induction was conducted in murine and human cell lines so real-time PCR standard curves where prepared for both murine and human IFN-β genes. 18SrRNA was used as a housekeeping gene in both sets of experiment, and as the region amplified has an identical sequence in both species the same set of standards were used for both. An initial round of PCR was conducted with each primer set and the PCR product purified by use of the QIAquick PCR purification kit. The concentrations of these products were then determined by the NanoDrop 100 Spectrophotometer (Thermo Fisher Scientific) and serial ten-fold dilutions of cDNAs made according to mass or copy number. Figure 5.3 shows the standard curves for the real-time PCRs of murine IFN-β, human IFN-β and 18SrRNA along with melt curve analysis deomstrating each PCR produced a single specific product at a satisfactory reaction efficiency. During quantitative PCR reactions, standard curves made of five points in the same concentration range as the samples were used. Results were expressed as copies of IFN-β per 100 copies 18SrRNA.

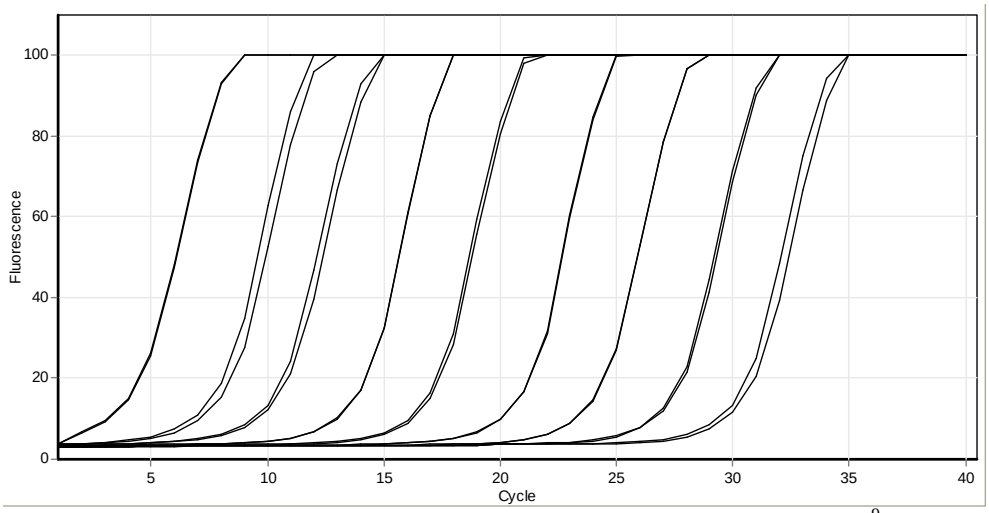


Figure 5.3 B(a) Standard curve of human IFN-β; ten-fold dilutions from 5.8 x 10^9 copies / µl to 5.8 x 10^1 copies/µl

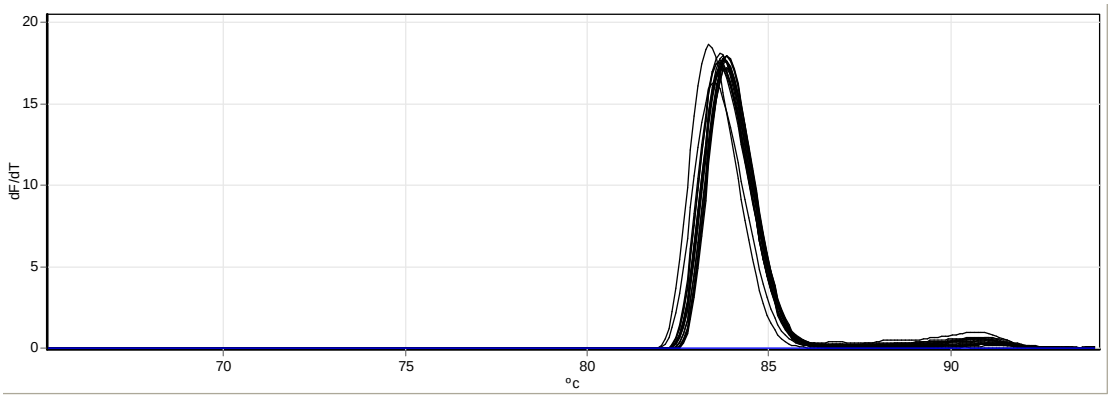


Figure 5.3 B(b) Melt curve analysis of human IFN-β qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	1.01862 (* = 10^(-1/m) - 1)
M	-3.2781
B	1.1734
R Value	0.99942
R^2 Value	0.99885

Figure 5.3 B(c) Efficiency data of human IFN-β qPCR

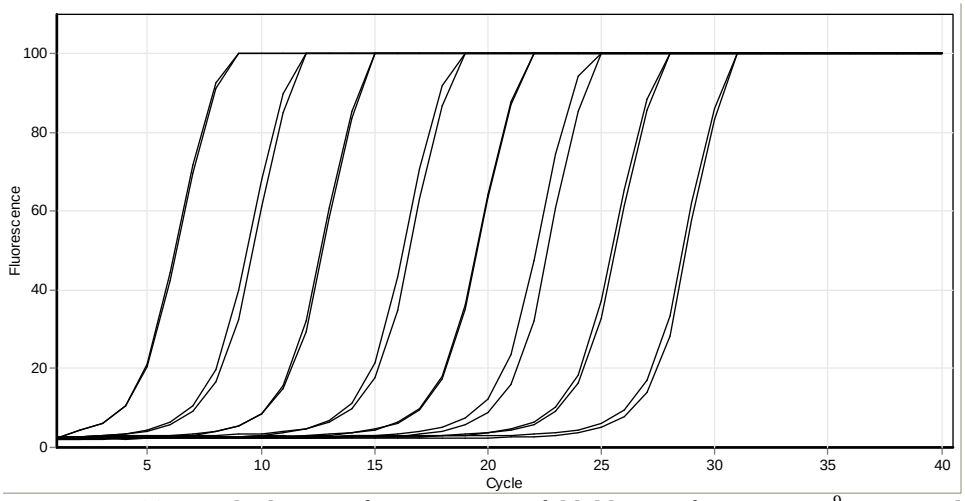


Figure 5.3C (a) Standard curve of 18SrRNA; ten-fold dilutions from 5.8 x 10^9 copies / µl to 5.8 x 10^2 copies/µl.

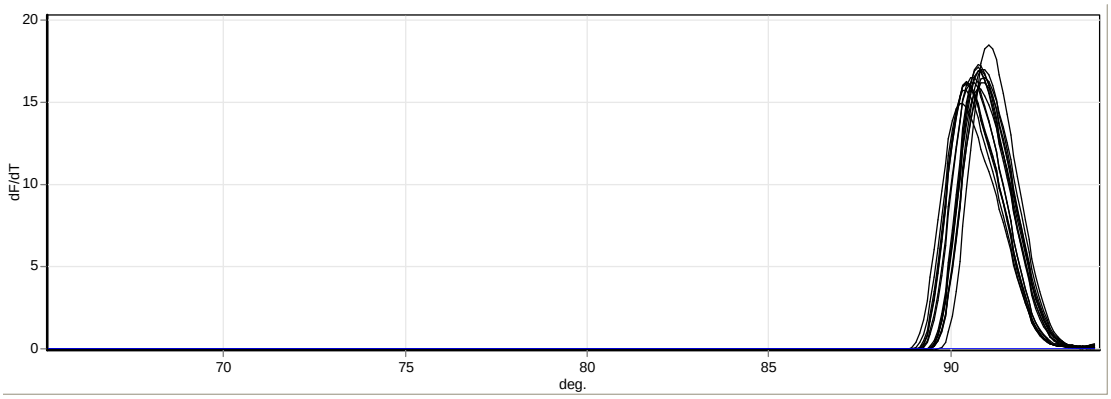


Figure 5.3 C(b) Melt curve analysis of 18SrRNA qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	1.06811 (* = 10^(-1/m) - 1)
M	-3.16883
B	30.00549
R Value	0.99948
R^2 Value	0.99896

Figure 5.3 C(c) Efficiency data of 18SrRNA qPCR

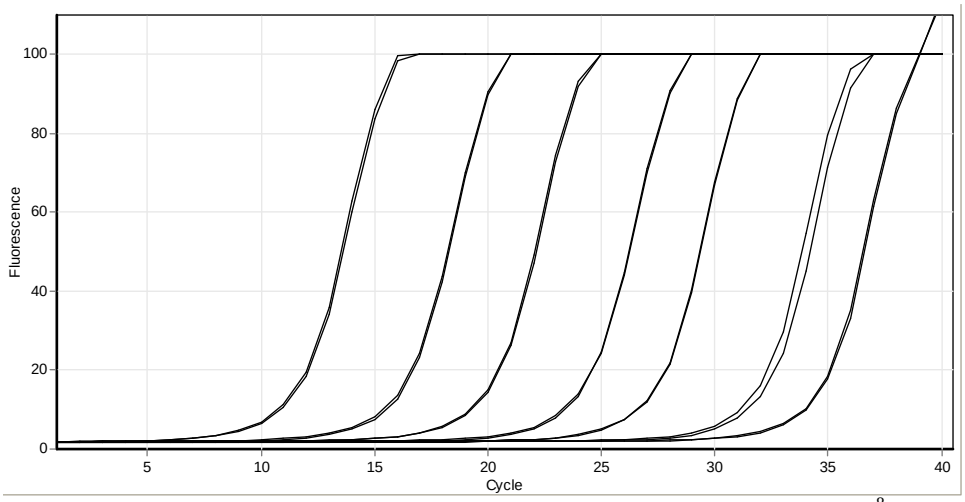


Figure 5.3A (a) Standard curve of murine IFN-β; ten-fold dilutions from 6.1 x 10^8 copies / µl to 6.1 x 10^2 copies/µl

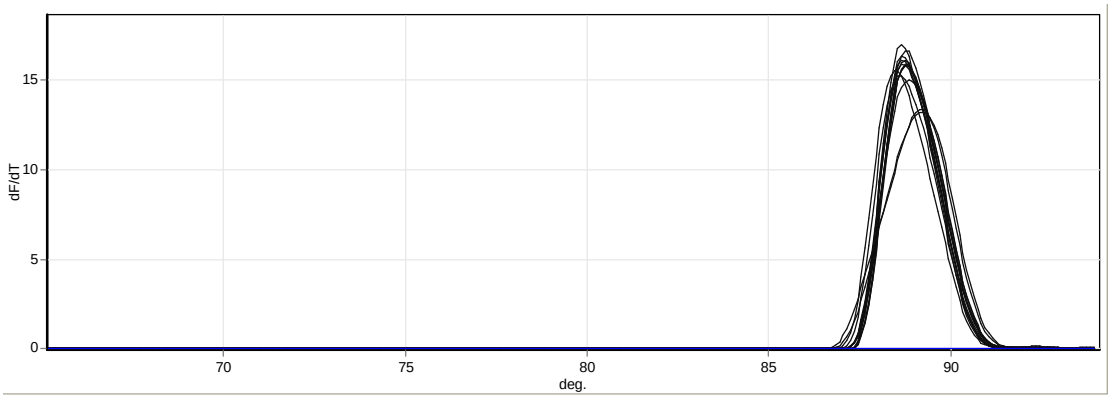


Figure 5.3 A(b) Melt curve analysis of murine IFN-β qPCR; single prominent peak indicates a single specific PCR product

Reaction efficiency (*)	0.87036 (* = 10^(-1/m) - 1)
M	-3.67749
B	13.03386
R Value	0.99873
R^2 Value	0.99745

Figure 5.3 A(c) Efficiency data of murine IFN-β qPCR

12-well plates were seeded at a density of 1.0 $x10^5$ cells per well. Approximately 24 hours later, 500 ng of each long (full length or truncated, see table5.2) viral RNA transcript was transfected into NIH3T3 cells; poly I:C was used as a positive control and mock cells were untreated. 18 hours post-transfection the cells were collected for analysis by real-time PCR. In the luciferase studies, 24-well plates were seeded with 5.0 x 10^4. Approximately 24 hours later, the IFN-β promoter-controlled firefly luciferase (p125-Luc) and, as an internal control, renilla (pRL-CMV), reporter plasmids were transfected into the cells, 24 hours post-transfection of the plasmids, the transcripts were transfected into the cells. The cells were lysed 18 hours post-transfection for analysis; mock cells were untreated, and wells were transfected with the luciferase plasmids alone. Both experiments gave a remarkably similar result, the GORS viruses induced IFN-β only mildly above mock; the non-GORS viruses were variable in their strength of induction, BV, PV and TMEV all induced strongly, whereas RV and SFV did not induce at all. The real-time PCR result is shown in figure 5.4, luciferase reporter assay result in figure 5.5.

The same experiments were repeated, with 500 ng of the long transcripts transfected into A549 cells with a similar result. The GORS viruses HCV and MNV induced IFN-β weakly, while GBV-C and PEMV2 did not induce a significant response above mock; of the non-GORS viruses, BV, PV and TMEV induced strongly, RV very weakly and SFV did not induce. The real-time PCR result is shown in figure 5.6, luciferase result in figure 5.7.

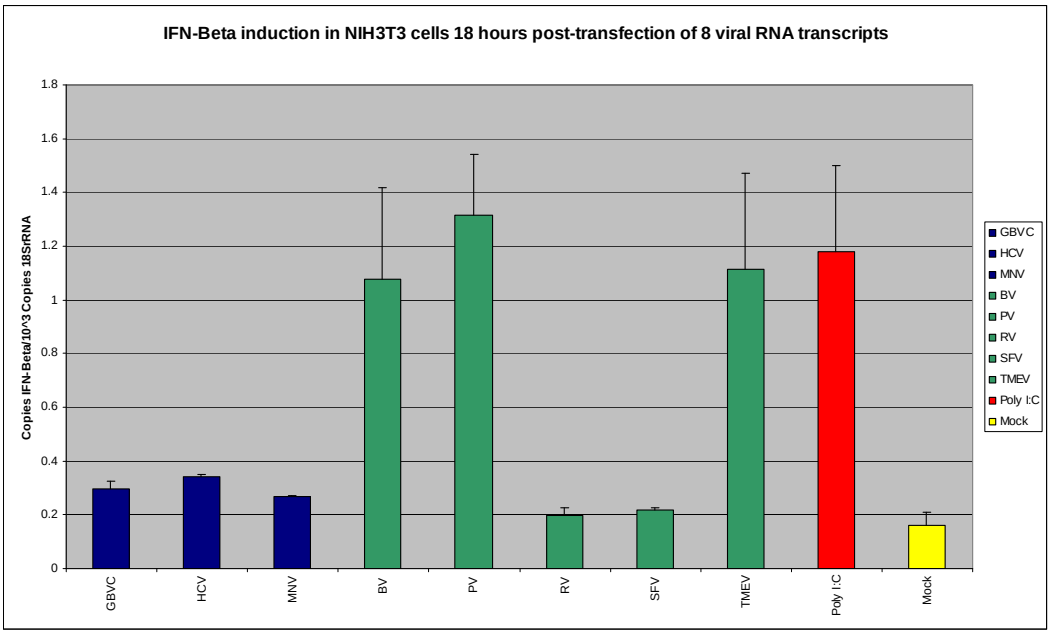


Figure 5.4 IFN-β induction in NIH3T3 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts measured by quantitative real-time PCR; transcripts were tested in duplicate; error bars are standard deviations of the mean.

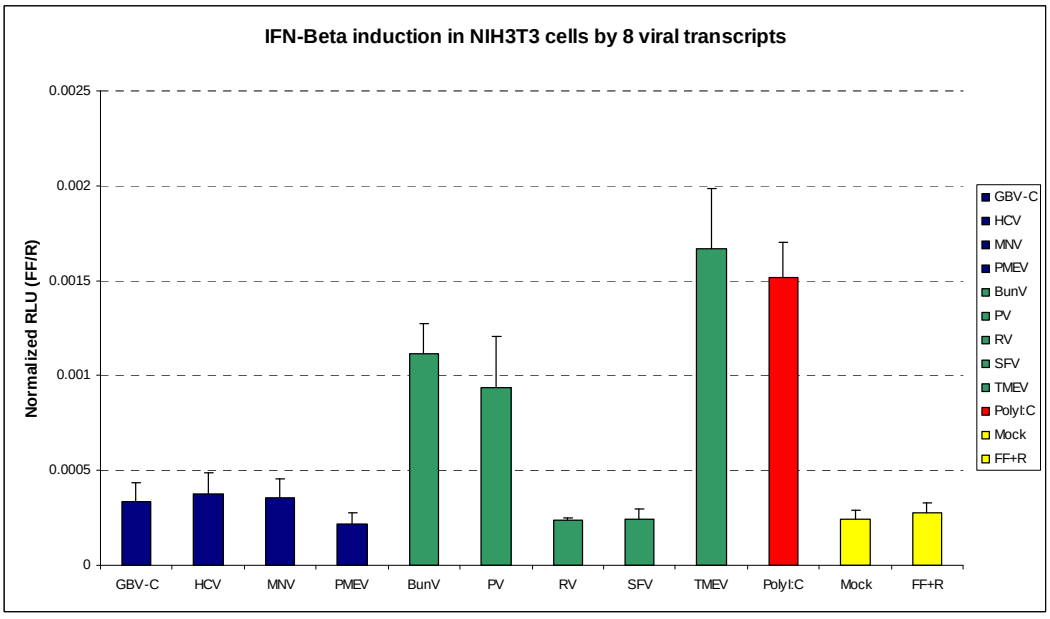


Figure 5.5 Expression of luciferase reporter in NIH3T3 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts; transcripts were tested in triplicate, error bars are standard deviations of the mean. BV, PV and TMEV are significantly increased over mock, whereas GBV-C, HCV, MNV, PEMV, RV and SFV are not ($P < 0.05$, unpaired t test)

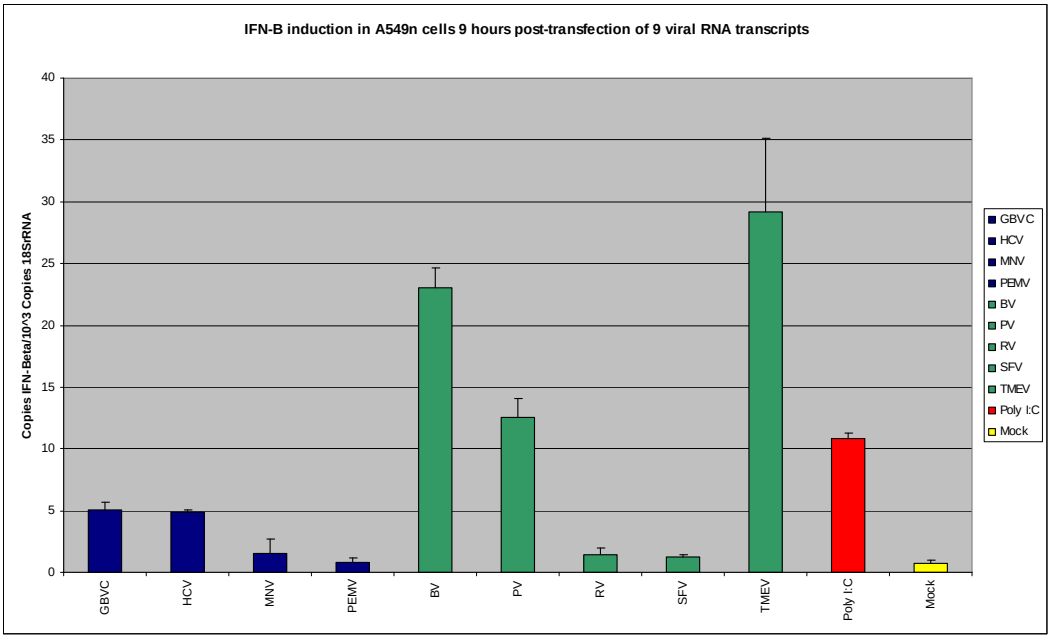


Figure 5.6 IFN-β induction in A549 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts measured by quantitative real-time PCR; transcripts were tested in duplicate; error bars are standard deviations of the mean.

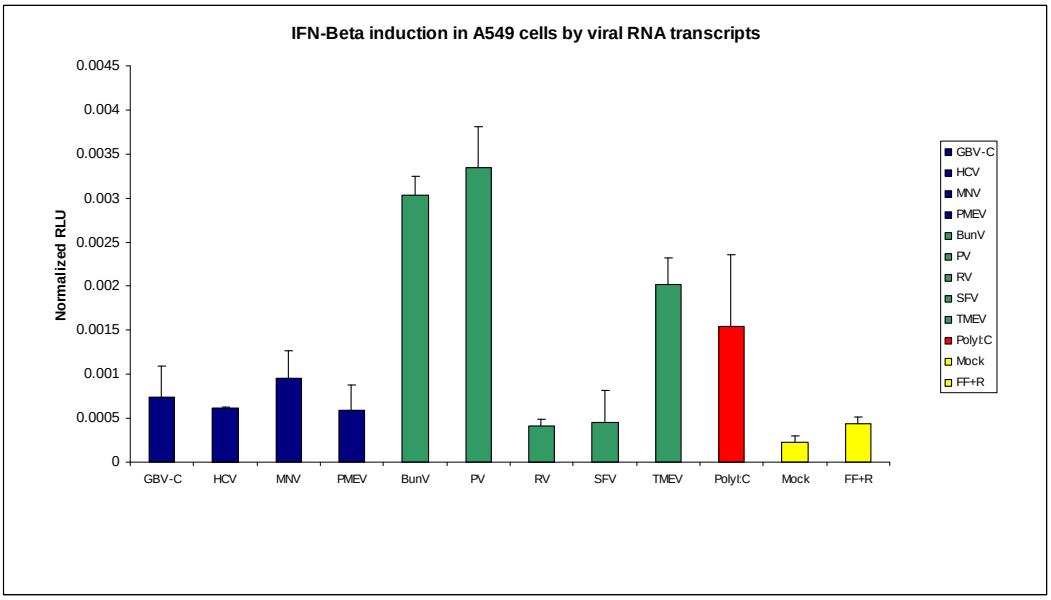


Figure 5.7 Expression of luciferase reporter in A549 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts; transcripts were tested in triplicate; error bars are standard deviations of the mean. All transcripts induced a significant response above mock but for GBV-C and SFV; but BV, PV and TMEV induced a significantly greater response than all other transcripts ($P < 0.05$, unpaired t test

5.3.3 IFN-β induction by GORS and non-GORS viral RNA transcripts pre- and post-phosphatase treatment.

The PRRs responsible for the cytoplasmic detection of dsRNA are RIG-I and MDA-5. Although both these receptors have been shown to respond to poly I:C (Rothenfusser *et al*., 2005; Yoneyama *et al*., 2005; Gitlin *et al*., 2006), a synthetic dsRNA analogue, the specific ligands in nature for each receptor are different. The ligand recognised by RIG-I has recently been shown to be the 5' triphosphate group (Hornung *et al*., 2006; Pichlmair *et al*., 2006). There is still uncertainty about the precise nature of the ligand for MDA-5, but it has been shown to detect dsRNA of length greater than 1 kilobase, whereas RIG-I detects shorter RNAs. In order to investigate the possible roles of RIG-I and/or MDA-5 the full length/truncated viral RNA transcripts they were treated with Antarctic Phosphatase (New England Biolabs).

The IFN-β induction experiments were repeated: in NIH3T3 cells, IFN-β induction was measured by real-time PCR and in A549 cells by luciferase reporter assay. All methods and conditions as previously described. The real-time PCR result is shown in figure 5.8, the luciferase reporter assay result in figure 5.9. In both experiments, both dephosphorylated transcripts and untreated transcripts were used so a direct comparison could be made between the two groups. In both studies the effect of dephosphorylation was that none of the transcripts induced an IFN-β response. It may be noted that the untreated transcripts produced the same pattern of induction as previous experiments with none of the GORS transcripts inducing strongly and of the non-GORS transcripts, BV, PV and TMEV induced strongly but RV and SFV did not induce an IFN-β response.

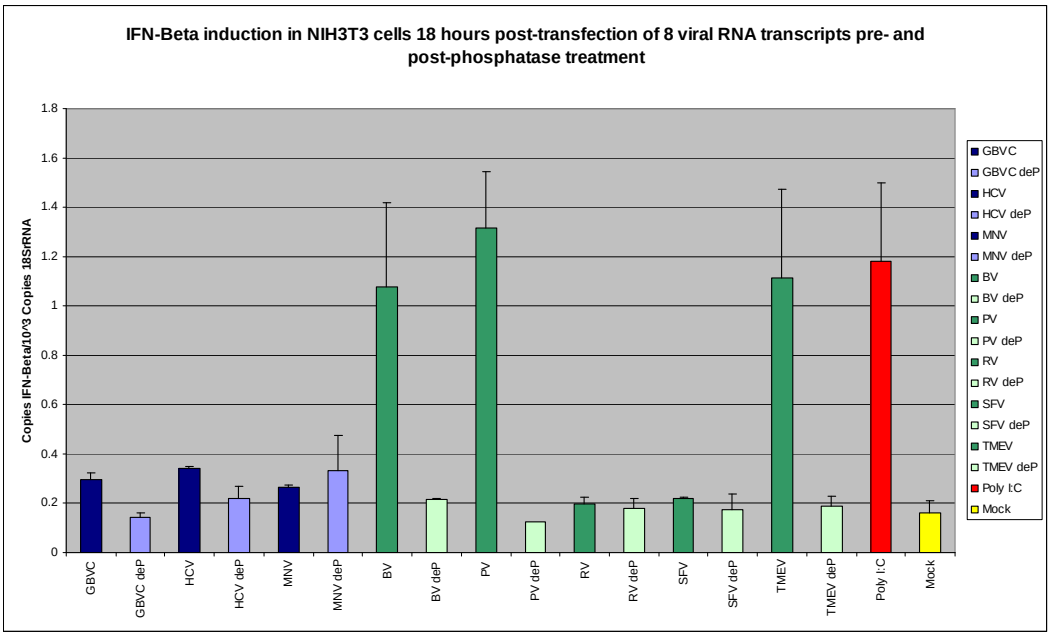


Figure 5.8 IFN-β induction in NIH3T3 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts, phosphorylated (dark blue or green bars) and dephosphorylated (light green and blue bars) measured by quantitative real-time PCR; transcripts tested in duplicate; error bars are standard deviations of the mean

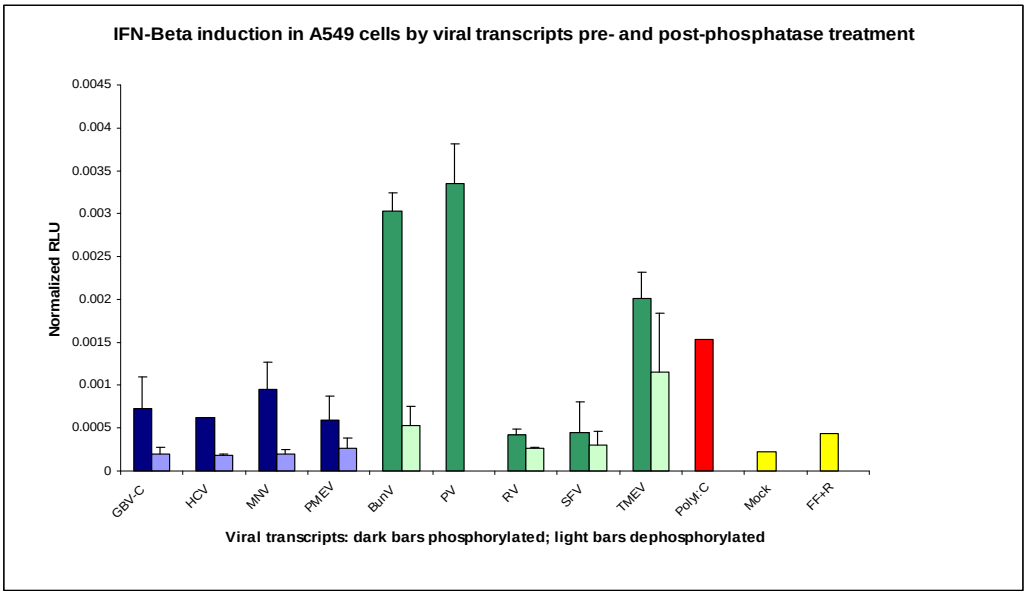


Figure 5.9 Expression of luciferase reporter in A549 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts, phosphorylated (dark blue or green bars) and dephosphorylated (light green and blue bars) transcripts tested in triplicate; error bars are standard deviations of the mean. Phosphorylated transcripts as in figure 5.7. No dephosphorylated transcripts induced a significant response above mock ($P < 0.05$, unpaired t test). Dephosphorylated BV induced significantly less strongly than BV, although there was no significant difference between TMEV pre- and post- dephosphorylation ($P < 0.05$, unpaired t test). Dephosphorylated PV transcripts were not tested during this experiment)

5.3.4 IFN-β induction by GORS and non-GORS transcripts in A549 and A549 NPro cells

Bovine viral diarrhoea virus, a pestivirus of the family *Flaviviridae*, produces the protein NPro which inhibits the production of IFN by blocking the activity of IRF-3 (Hilton *et al*., 2006). In an A549 cell line expressing this protein the IFN induction experiment was repeated with a single GORS and non-GORS virus, GBV-C and TMEV, with poly I:C as a positive control. This cell line was used to investigate if the IFN-β response seen in our studies so far was via the IRF-3 pathway. The experiment was conducted with A549n cells simultaneously with A549 NPro cells; the degree of IFN-β induction measured by real-time PCR. Figure 5.10 shows that whereas the A549n cells responded as before i.e. weak induction by GBV-C and strong induction by TMEV, there was no IFN-β induction at all in the A549 NPro cells, even with the positive control poly I:C.

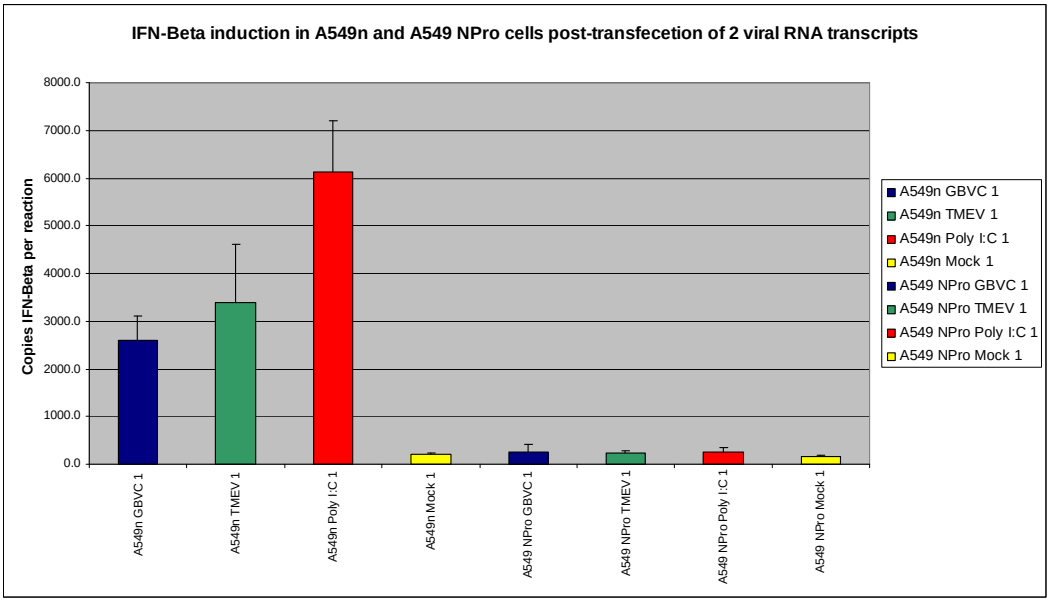


Figure 5.10 IFN-β induction in A549n and A549 NPro cells following transfection of a predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcript, phosphorylated measured by quantitative real-time PCR.

5.3.5 IFN-β induction by 4 kb GORS and non-GORS transcripts

The longer transcripts used in these studies up to this point had given a consistent result in two cell lines and by two different methods conducted independently. The differing strengths of IFN-β induction across the panel of viruses was an interesting finding, although the failure of RV and SFV to induce a strong response meant that there was not a simple correlation between strength of induction and the presence of GORS (to be discussed in more detail later). One potential factor that may have caused the long transcripts to behave differently was transcript length, the transcripts are of different lengths from 4200 – 9236, so that the same mass of RNA that was transfected in each experiment i.e. 500 ng, would contain different copy numbers of RNA molecules. The exact nature of the form that GORS RNA takes as opposed to non-GORS RNA remains somewhat uncertain, although atomic force microscopy studies suggest that GORS RNA transcripts adopt tightly bound globular structures whereas non-GORS transcripts appear extended (Davis *et al*., 2008). With these factors in mind, it was not certain that these other variables were not having an effect on the strength of IFN-β induction. Another potential factor was the possibility of translation. None of the transcripts was replication competent as those that were truncated were missing the 3' end of their genomes. Some of the viruses, the flaviviruses (GBV-C, HCV) and picornaviruses (PV, TMEV), have a 5' UTR that contains an IRES so could, possibly, undergo translation and produce viral proteins, which could have an effect on IFN-β induction e.g. HCV NS3 inactivates Cardif/VISA/MAVS.IPS-1 (Li *et al*., 2005). For these reasons transcripts were made of 4 kilobases (kb) in length from regions of the genome that did not include the 5' UTRs of the flaviviruses or picornaviruses. 1×10^{11} copies, or ~ 220 ng, of each 4 kb transcripts was transfected into NIH3T3 cells. Figure 5.11 shows a preliminary experiment involving transfection of the long transcripts and 4 kb transcripts of GBV-C and TMEV in NIH3T3 cells, measured by real-time PCR. The long GBV-C and TMEV transcripts behave similarly to experiments already described i.e. little/no induction with GBV-C and strong induction with TMEV. The result with the 4 kb transcripts is similar in that GBV-C still does not induce and TMEV does, but the response appears weaker, although not statistically

significant as the large mock value in this particular experiment prevents meaningful analysis.

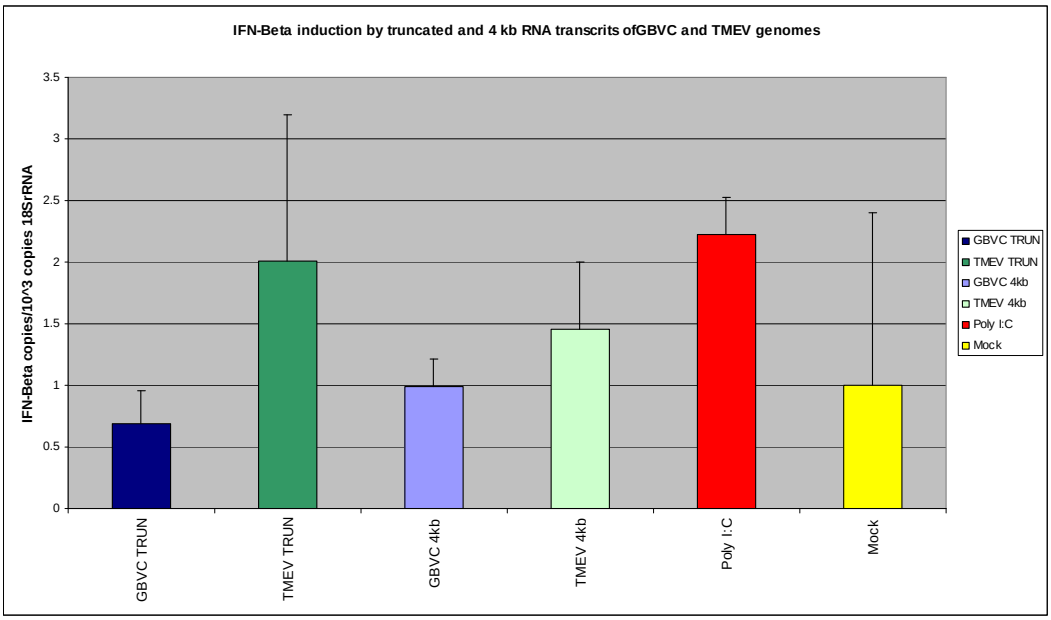


Figure 5.11 IFN-β induction in NIH3T3 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts, long transcripts (dark blue/ green bars) and 4 kb transcripts (light green/blue bars) measured by quantitative real-time PCR.

This experiment was repeated with the panel of viruses in NIH3T3 cells and A549 cells using the luciferase reporter assay. Figure 5.12 shows IFN-β induction with long and 4 kb transcripts in NIH3T3 cells. The long transcripts induced in the same pattern as seen in all previous experiments, i.e. significant induction by BV, PV and TMEV, but by none of the other transcripts. The 4 kb transcripts of BV and PV are the strongest inducers, but the induction is significantly weaker than in the long transcripts. Only the 4 kb transcript of BV induces IFN to a comparable degree to that of the long transcript. There is little difference between the long and 4 kb transcripts in those viruses that do not induce a strong response in with either long or 4 kb transcripts.

Figure 5.13 repeats the experiment in A549 cells, and while the long transcripts have the same pattern as in previous studies, the 4 kb transcripts do not. All of the 4 kb transcripts have induced a response higher than mock with the exception of PEMV2 With the exception of BV, there is little difference in the level of luciferase reporter expression between any of the viruses, GORS or non-GORS. The 4 kb transcripts of RV and SFV have even produced a higher signal than their longer counterparts. The 4 kb transcripts were also treated with phosphatase, the transfection repeated and IFN induction measured by luciferase assay, the result is shown in figure 5.14. As in the long transcripts, phosphatase treatment markedly reduces or eliminates IFN induction by most of the transcripts.

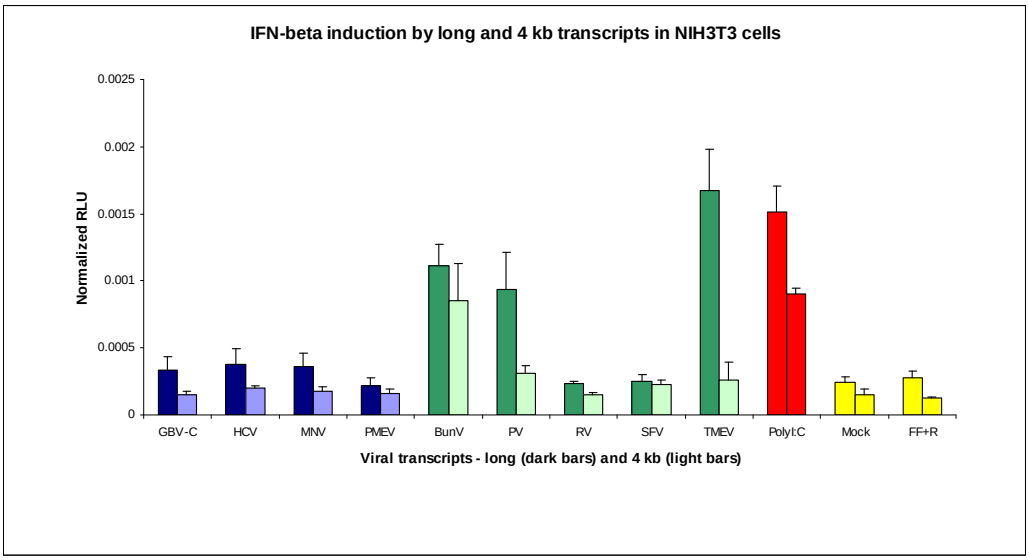


Figure 5.12 Expression of luciferase reporter in NIH3T3 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts, long transcripts (dark blue/ green bars) and 4 kb transcripts (light green/blue bars); transcripts tested in triplicate; error bars are standard deviations of the mean. Long transcripts of BV, PV and TMEV induced a significant response over mock, the other transcripts did not ($P < 0.05$, unpaired t test); 4 kb transcripts of BV, PV and TMEV induced a significant response over mock, the other transcripts did not ($P < 0.05$, unpaired t test); the response to the 4 kb transcripts was significantly lower with the 4 kb transcripts of PV and TMEV compared to long ($P < 0.05$, unpaired t test).

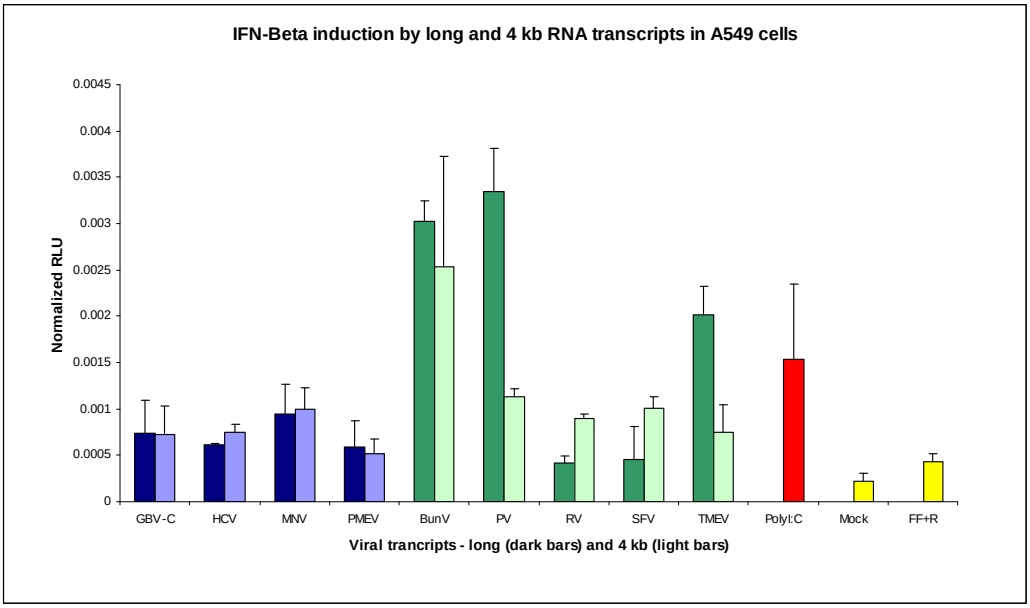


Figure 5.13 Expression of luciferase reporter in A549 cells following transfection of predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts, long transcripts (dark blue/ green bars) and 4 kb transcripts (light green/blue bars); transcripts tested in triplicate; error bars are standard deviations of the mean. All long and 4 kb transcripts induced a significant response over mock except for PEMV, 4 kb and long, and SFV long. ($P < 0.05$, unpaired t test); 4 kb transcripts of RV and SFV induced a significantly greater response than long ($P < 0.05$, unpaired t test); long transcripts of BV, PV and TMEV induced a significantly stronger response than other long transcripts, but there was no significant difference in degree of induction across the 4 kb transcripts($P < 0.05$, unpaired t test).

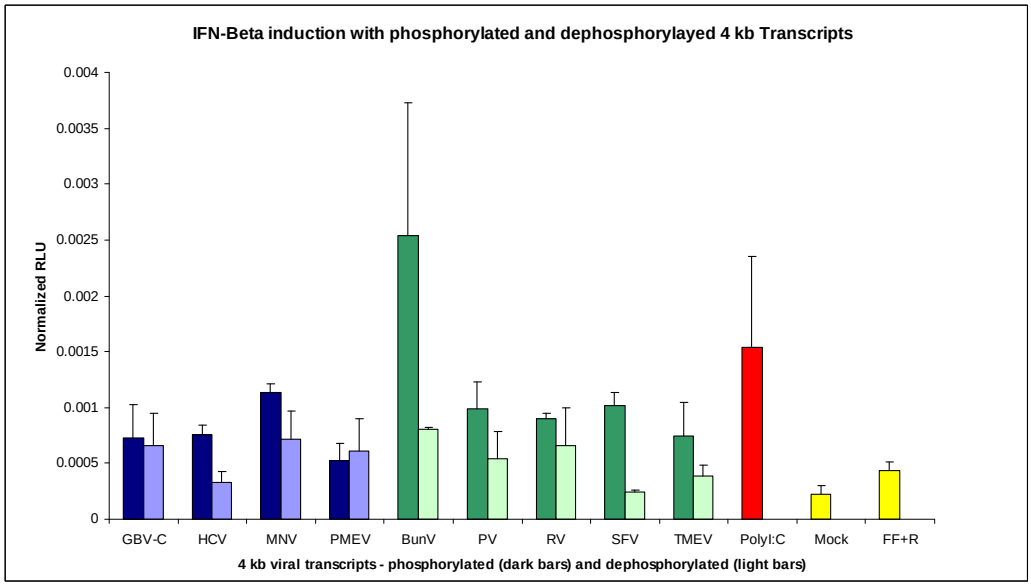


Figure 5.14 Expression of luciferase reporter in A549 cells following transfection of 4 kb transcripts predicted GORS (blue bars) and non-GORS (green bars) viral RNA transcripts, phosphorylated (dark blue/ green bars) and dephosphorylated transcripts (light green/blue bars); transcripts tested in triplicate; error bars are standard deviations of the mean. All phosphorylated 4 kb transcripts induced a significant response above mock but for PEMV ($P < 0.05$, unpaired t test); dephosphorylated transcripts of GBV-C, MNV and BV induced a significant response above mock, the others did not ($P < 0.05$, unpaired t test); there was a significant difference between the strength of signal by phosphorylated and dephosphorylated transcripts in HCV, MNV, BV and SFV, but not the others ($P < 0.05$, unpaired t test).

5.3.6 IFN-β induction: dose response to long and 4 kb RNA transcripts

With the pattern of IFN-β induction consistent throughout the panel of GORS and non-GORS viral RNA transcripts used in this study, the next step in this study was to perform a dose response experiment of both the long and 4 kb transcripts. For this experiment, the amount of long transcripts transfected into the cells was based on copy number rather than mass. The previous experiments had used 500 ng of each transcript transfected per well, which was equivalent to 1×10^{11} copies of the GBV-C transcript. Taking 1×10^{11} copies as a starting point, the following copy numbers of each transcript were transfected into NIH3T3 cells: 2.2×10^{11}, 1×10^{11}, 4.4×10^{10}, 2×10^{10} and 8.8×10^{9} copies. Figure 5.15 shows that the BV transcript copy numbers that induce the most strongly are ,1×10^{11} and 4.4×10^{10}, higher and lower copy numbers induces less strongly; copy number has little effect on the degree of IFN induction by GBV-C i.e. there is little or no induction. Figure 5.16 shows the first three copy numbers inducing strongly, but the strength of the signal declines thereafter. A similar dose response study was conducted with the 4 kb transcripts. Figure 5.17 shows a dose response from 1×10^{11}, 4.4×10^{10} and 2×10^{10} copies of GBV-C, MNV and BV measured by real-time PCR; figure 5.18 shows a dose response from 5×10^{10}, 3.8×10^{10}, 2.5×10^{10}, and 1×10^{10} copies measured by luciferase assay. The results are similar to those seen in the longer transcripts in that as the copy number is reduced, so is the level IFN-β induction from by the transcript previously shown to be an inducer. There is no effect by altering the copy number on the ability to induce an IFN response in those transcripts that have never previously shown that capability.

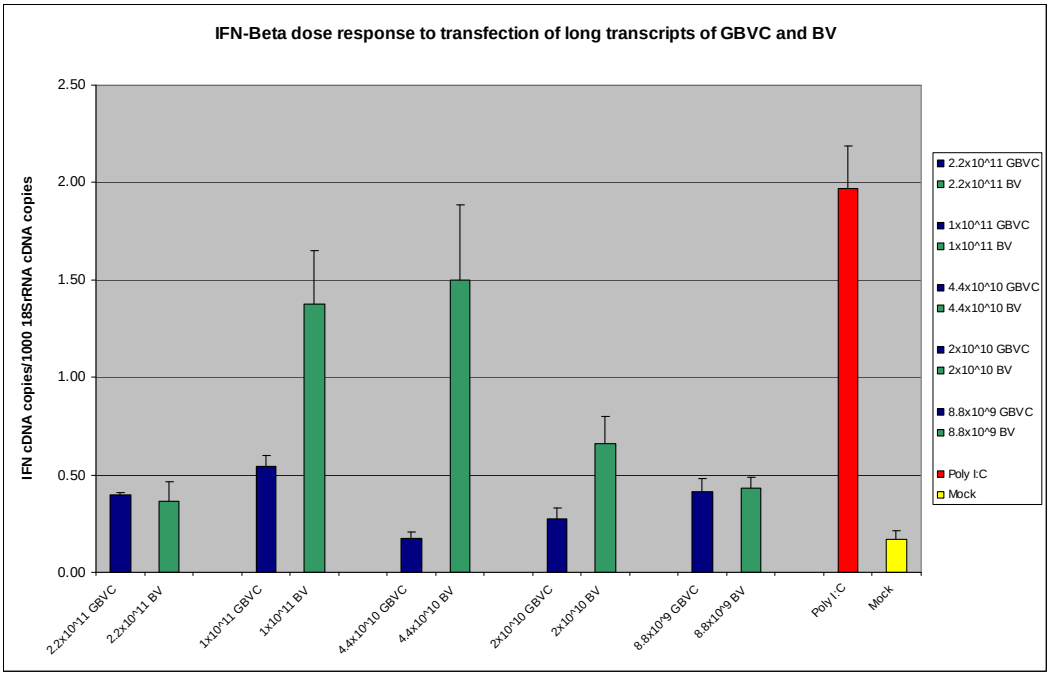


Figure 5.15 IFN-β induction in NIH3T3 cells – dose response to long transcripts of GBV-C (blue bars) and BV (green bars) RNA transcripts measured by real-time PCR; each time point was analysed in triplicate; error bars are standard deviations of the mean. The first, second and fifth copy numbers of GBV-C induced a significant response above mock, but there is no apparent pattern according to copy number; BV induced a significant response at all copy numbers but was significantly higher than GBV-C from 1 x 10^{11} to 2 x 10^{10} ($P < 0.05$, unpaired t test).

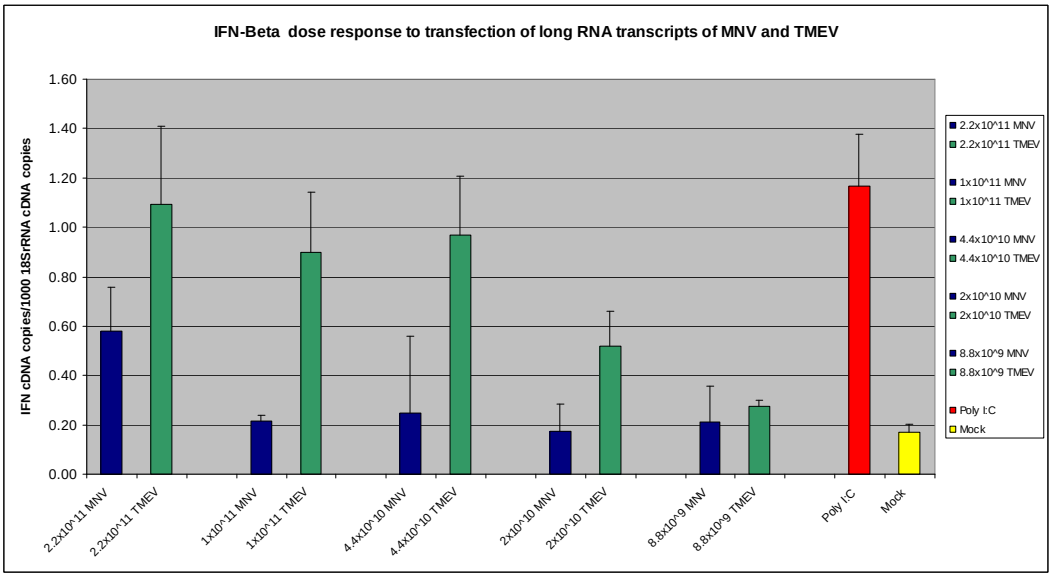


Figure 5.16 IFN-β induction in NIH3T3 cells – dose response to long transcripts of MNV (blue bars) and TMEV (green bars) RNA transcripts measured by real-time PCR; each time point was analysed in triplicate; error bars are standard deviations of the mean. Only the highest copy number of MNV induced a significant response above mock, whereas all copy numbers of TMEV did and was significantly higher than MNV from 1 x 10^{11} to 2 x 10^{10} ($P < 0.05$, unpaired t test). There is a declining trend of strength of induction asa

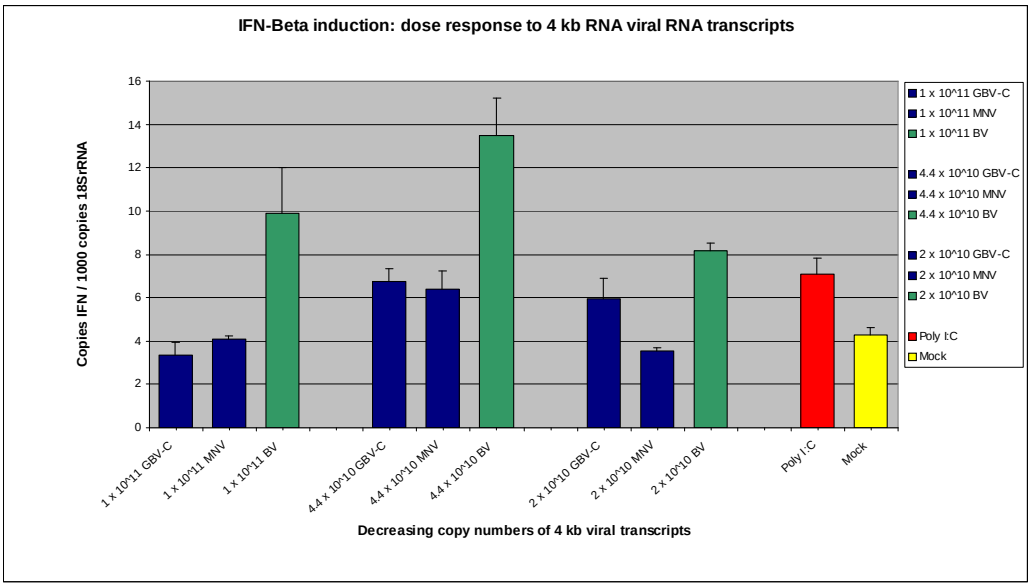


Figure 5.17 IFN-β induction in NIH3T3 cells – dose response to 4 kb transcripts of GBV-C, MNV (blue bars) and BV (green bars) RNA transcripts measured by real-time PCR; ; each time point was analysed in triplicate; error bars are standard deviations of the mean. GBV-C and MNV only induced a significant response at 4.4 x 10^{10} copies, whereas BV induced a significant response at all copy numbers and induced a significantly greater response than GBV-C or MNV at all copy numbers ($P < 0.05$, unpaired t test).

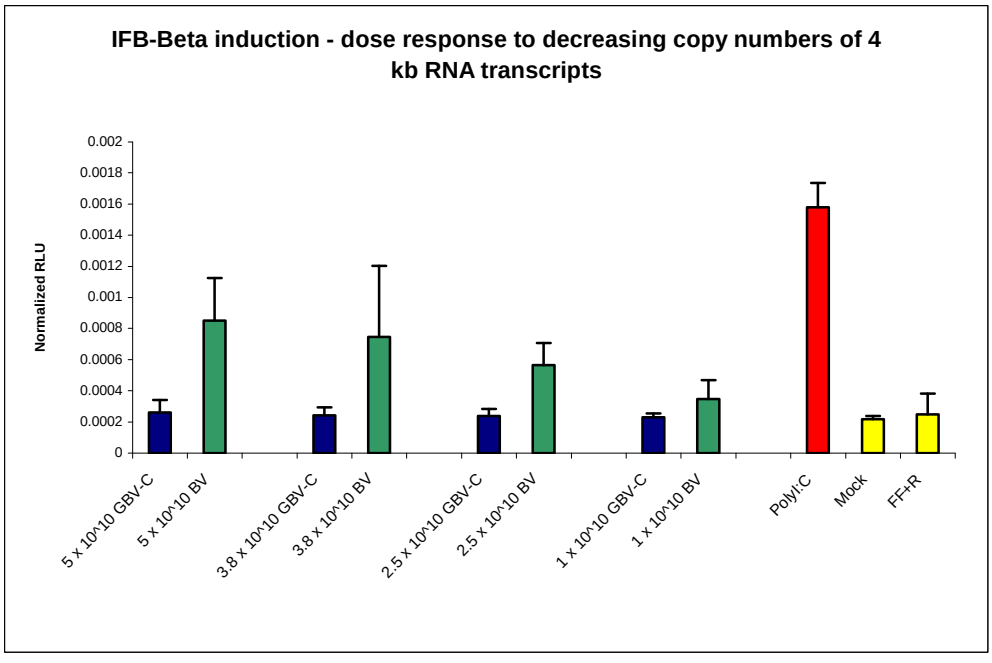


Figure 5.18 Expression of luciferase reporter in NIH3T3 cells – dose response to 4 kb transcripts of GBV-C (blue bars) and BV (green bars) RNA transcripts; transcripts tested in quadruplicate; error bars are standard deviations of the mean. GBV-C did not induce a significant response at any copy number, whereas BV induced a significant response at every copy number and induced a significantly greater response than GBV-C at all copy numbers except 1 x 10^{10} ($P < 0.05$, unpaired t test).

5.4 Discussion

RNA plays a vital role in biology; eukaryotic cells contain their genetic information in dsDNA, but RNAs are essential in the transcription and translation of those genes into proteins. It has also been shown in recent years that the regulation of RNA production and degradation is an important factor in the regulation of gene expression (reviewed by Garneau *et al*., 2007). RNA also makes up the genetic makeup of many viruses that parasitize cells. The RNA genomes, if they are positive sense, can be translated directly by host ribosomes; negative sense genomes have to undergo a round of transcription to create a positive sense RNA molecule. Cellular mRNAs have 5' caps by which they interact with ribosome, and while some RNA viruses also have 5' caps, others interact with ribosomes through conserved secondary structures such as the IRES and CRE. These structures generally consist of stem-loops of 200 -500 nucleotides of extensive internal base-pairing (Belsham and Sonenberg, 1996) During their replication, RNA viruses also generally go through stages where dsRNA is generated in forms such as replicatory intermediates. dsRNA is foreign to cells and is the most important of the PAMPs that viruses generate (Marcus, 1983).

The discovery of GORS (Simmonds *et al*., 2004) revealed the presence of extensive secondary structure throughout the genomes of some genera of positive sense RNA viruses, and an observation was made that this phenomenon was associated with viruses able to establish persistent infection in their natural hosts. The studies presented here are an investigation into the possible mechanisms by which GORS leads to persistence. We focused on the interaction of RNA transcripts of GORS and non-GORS viruses with the mechanisms of cellular RNA stability and the induction of IFN-β. We hypothesised that the presence of GORS would lead to greater RNA stability in transcripts than those transcripts without GORS; and that GORS viral genomes would induce IFN-β less strongly than non-GORS viruses. IFN-β induction studies were carried out independently by two methods, by real-time quantitative PCR and by a luciferase reporter assay; the findings of these studies are summarised below:

Summary of findings

- There is no significant difference in the intra-cellular stability of GORS and non-GORS viral RNA transcripts

- Of the panel of long transcripts of GORS and non-GORS viruses used in IFN-β induction studies, none of the GORS viruses (GBV-C, HCV, MNV, PEMV2) induced IFN-β strongly; but there was variable induction from the non-GORS viruses, BV, PV and TMEV inducing strongly but SFV and RV inducing very weakly or not inducing at all.

- Dephosphorylation of the RNA transcripts greatly reduced or eliminated the IFN response

- Transfection of transcripts into A549 NPro cells resulted in no IFN-β induction

- Transfection of translation incapable, 4 kb RNA transcripts into NIH3T3 cells revealed the same pattern of induction across the same panel of viral transcripts, although there was a reduced degree of IFN-β induction compared to the longer transcripts.

- Transfection of translation incapable, 4 kb RNA transcripts into A549 cells revealed that only BV induced significantly more strongly than the other viruses, and that there was little difference in the strength of induction between the other viruses.

- There was a dose response in IFN-β expression according to copy number of RNA transcripts transfected

Viral RNAs may encounter degradation by the RNase L system, an IFN stimulated response in which viral and cellular RNAs are degraded. They may also encounter the cellular exonucleases that have a role in cellular RNA stability and regulation of gene expression. These cellular exonucleases are likely to be the more significant enzymes that target viral genomic RNAs immediately after uncoating, before the IFN response produces large amounts of RNase L. The avoidance of RNA decay machinery by viral RNAs can be split into two strategies: modulation of the host decay mechanisms and physical avoidance of the decay mechanisms (Sokolski *et al*., 2006). During replication, RNA viruses often incorporate cellular elements and viral ribonucleoprotein (RNP) into replication complexes. These complexes may be sufficient to shield replicating viruses from cellular exonucleases. Just as significantly, they may shield viruses from intracytoplasmic detectors of dsRNA which induce an IFN response; and the IFN-stimulated 2'5'OAS activates RNase L which degrades cellular and viral RNAs. Before establishing replication complexes, virus must uncoat their genomes, thus exposing their genomic RNAs to the intracellular environment. The study presented here seeks to determine whether structured RNAs are more stable than unstructured RNA after electroporation into NIH3T3 cells. The result is that there is no difference in the rate of decay of the three GORS viral transcripts (GBV-C, HCV, MNV) and the three non-GORS transcripts (BV, PV, TMEV). These six viruses belong to different virus families and naturally infect different cell type in different hosts, but what this study shows is that in the same cell type, under the same conditions, the degree of secondary structure alone confers no protection against cellular RNA decay mechanisms. The cellular exonucleases would seem to be unaffected by the presence of stem-loops or the more globular structure of the GORS transcripts as observed by atomic force microscopy (Davis *et al*., 2008). During natural infections by the viruses tested in this study other factors come into play concerning the capacity to avoid RNA degeneration. The 3' UTR of HCV binds to the cellular protein HuR, resulting in protection against exonucleases (Spanberg *et al*., 2000). Based on this study, it seems unlikely that secondary structure has a large role to play in RNA stability.

The observation that GORS viruses establish persistent infections in their natural hosts is one of the most intriguing aspects of the in original description of GORS. GORS itself appeared to be a conserved feature of genomes across virus families, and the association with persistence was likewise maintained across virus families. Initial speculation as to the mechanism behind this ability to persist focused on the innate cellular immune system i.e. type I IFNs. dsRNA has been known to be the most important viral inducer of IFN for many years (Marcus, 1983) so it was hypothesised that the presence of GORS would in some way limit the exposure of viral RNA to reduce the level of IFN induction. The induction of IFN-β by viral RNA transcripts in these studies produced a consistent result in repeated experiments using two different experimental methods to measure IFN-β induction. The transcripts were made to be replication incapable so that the effect was limited to the RNA molecules transfected. None of the long GORS viral transcripts (GBV-C, HCV, MNV, PEMV2) induced IFN-β strongly; whereas of the non-GORS transcripts, BV, PV and TMEV consistently induced a strong IFN-β response, but RV and SFV did not. This result is interesting as, although is does not confirm the hypothesis, it raises more interesting questions. Firstly, it is still possible that the presence of GORS does have a protective effect but that the RV and SFV transcripts have other properties that prevent them from inducing a strong IFN response. The inclusion of PEMV2, a GORS virus that did not induce IFN, is that it is a plant virus, its natural host with which it has co-evolved is a plant, and it is able to parasitize plant cells. The main mechanism of innate immunity in plants is RNAi, so there is no reason to suppose that a plant virus would have evolved any mechanism at all to evade the IFN system of mammalian cells; but there was still no apparent recognition of its RNA as it did not induce IFN. An interesting addition to this study would have been a non-GORS plant virus to see if the NIH3T3 or A549 cells would have detected its RNA as foreign.

The lack of a strong response, or no response at all, with the long RV and SFV transcripts in either NIH3T3 cells or A549 cells, when the other non-GORS transcripts did induce a strong response was unexpected. It shows that although the panel of transcripts did induce IFN-β to different degrees, there was not a simple division between GORS and non-GORS. It is notable that both RV and

SFV are members of the virus family *Togaviridae*, and it would be interesting to repeat this experiment with other members of that family e.g. sindbis virus, eastern equine encephalitis virus, western equine encephalitis virus, Venezuelan equine encephalitis virus, chikungunya virus. This result is all the more surprising as SVF is known to induce an IFN response which acts to limits its spread (Fragkoudis *et al*., 2007; reviewed by Fazakerley, 2002). During SFV infection it has also been shown that PKR acts to enhance the IFN response (Barry *et al*., 2008). Alphaviruses, a genus of *Togaviridae*, are known to infect many cell types in many species; and NIH3T3 cells do produce an IFN response on infection with SFV (data not shown). Rubella virus has also been shown to induce an IFN response on infection of both adult and foetal human cells (Adamo *et al*., 2007). In this study, neither the SFV nor RV transcripts were of full length, as it was important to avoid RNA translation or viral replication. These truncated RNA transcripts may not have behaved as the genome does in actual infections, although the SFV and RV transcripts were of comparable length to the other transcripts in the panel, although in A549 cells the 4 kb transcripts of these two viruses appeared to lead to a greater IFN response. This failure of SFV and RV transcripts to induce an IFN response remains unexplained and requires further study, for example by use of SFV virus-like particles that contain defective genomes so cannot replicate.

The effect of dephosphorylating the transcripts was to markedly reduce or eliminate the IFN response. As RIG-I is the receptor that detects the 5' triphosphate group present on viral RNAs this result suggests that RIG-I is the main pattern recognition receptor detecting the transcripts in this study. In viral infections, those viruses that contain groups other than triphosphate on the 5' end of the genome e.g. the picornavirus or calicivirus VPg; the togavirus 7-methylguanosine cap, will not be detected by RIG-I at all, but by MDA-5. By necessity, to maintain the same experimental conditions the different transcripts had to be treated equally and each experiment conducted in a single cell type. Whilst the result of each experiment enables the comparison of the effect of the different transcripts in each cell type, NIH3T3 or A549, they do not allow a direct inference to be made to say that the same effect will be occurring during natural infection. In this study, the PV transcripts consistently induced a strong

IFN response, both the long truncated transcripts and the 4 kb transcripts, in NIH3T3 and A549 cells. With the dephosphorylated transcripts the effect was reduced or eliminated, suggesting a role for RIG-I, but during infection PV as a picornavirus is detected by MDA-5.

The experiments with 4 kb transcripts were conducted to control for differences in transcript length and the possibility of translation and production of proteins. The fact that in general the 4 kb GORS transcripts induced little or no IFN response in the NIH3T3 cells indicates that that lack of induction by the longer GORS transcripts in this cell line had not been a protein effect, and that the difference in IFN induction between the transcripts was almost certainly an RNA effect. In NIH3T3 cells, the viruses whose longer transcripts had induced the most strongly still produced the strongest signals with the 4 kb transcripts, although the level of IFN expression was generally lower. The difference in the response to longer and 4 kb transcripts may be a result of different level of access to the binding site of RIG-I. The RIG-I binding site has been recently described by Cui and colleagues. The C-terminal domain of RIG-I contains a regulatory domain (RD) which contains a zinc-binding domain which is the ligand binding site. The other RLRs have a similar structure, but in RIG-I there is a lysine residue at the centre of the binding site which is required for triphosphate binding, in MDA-5 in this position there is a threonine or isoleucine residue. The binding site of RIG-I is unable to detect dephosphorylated RNAs. RD is a flat domain, it has a concave and convex side, it has dimensions of 45 Å (4.5 nm) x 35 Å (3.5 nm) x 30 Å (3.0 nm) (Cui *et al*., 2008) Atomic forces microscopy has revealed that the transcripts of predicted GORS viruses GBV-C and HCV have a prolate spheroid shape. Their dimensions were, for GBV-C and HCV respectively, radii of 32 nm and 30 nm along the *x* and *y* axes, and mean heights of each transcript molecule of 3.9 ± 1.0 nm (Davis *et al*., 2008). With GORS transcripts forming tight clusters, it may be that the triphosphate groups are shielded from the binding site on the RIG-I RD. Figure 5.14 show the effect of dephosphorylation on 4 kb transcripts in A549 cells, and interestingly there is a small degree of IFN induction by the dephosphorylated transcripts which had not been seen in the NIH3T3 cells. Perhaps in this cell type there is a slightly contribution by MDA-5.

In A549 cells the 4 kb experiments had a different outcome. As can be seen in figures 5.13 and 5.14, in this cell line the there is no significant difference between the strength of induction across the panel of viruses. This is in contrast to the result in this cell line using the longer transcripts which is more similar to the result seen in NIH3T3 cells (see figures 5.4, 5.5, 5.6, 5.7). There may be a difference in the exact nature of the innate defences present in these two cell lines. Although the pathways of the IFN system may be shared by different cell types, it may be that different cell types within the body have evolved defence mechanisms that have been modified by exposure to viruses throughout evolution and have adapted to respond accordingly. Future work in this area could investigate the impact of different RNAs on all kinds of cell types, and reveal more valuable information about the relationship between cells and RNA viruses.

The dose response experiments have shown that the level of IFN induction is dose dependant. In many persistent infections the viruses continue to replicate at a low level, without lysing the cell and often are clinically silent. It would be interesting to establish the viral RNA copy numbers detectable during the actual infection by each of the viruses from this study. According to this study, below ~1 x 10^{10} viral copies per well (~5 x 10^{4} copies per cell) there was a barely significant IFN response. The transfection efficiency of Lipofectamine 2000 in these cells lines could have an effect on the outcome. Transfection efficiency is expressed as the percentage of cells to have been transfected, although it is not an indication of the exact mass of RNA to be delivered into the cell cytoplasm. These RNA copy numbers may be also misleading as the experiments were carried out in two cell types, NIH3T3 and A549, whereas in their natural hosts each viruses may have evolved a very particular replication strategy and innate immunity evasion strategy.

This study has investigated some of the complex relationships that exist between viral RNAs and cellular mechanisms. It is difficult to investigate the biological properties of the phenomenon of GORS as it exists across virus families that infect different hosts and different cell types. In attempting to control for these

variables in their design, the experiments are necessarily artificial. It is difficult to infer from these studies in cell culture what the mechanism is by which the presence of GORS in the genome leads to persistence during natural infection. It is an interesting finding that there was a consistent pattern of IFN induction across the panel of predicted GORS and non-GORS viral transcripts, and that none of the long GORS viruses induced. This could be the first indication of a mechanistic role for GORS in persistence, although the failure of long RV or SFV transcripts to induce remains unexplained. Further investigation is required to highlight where within the innate immune system the transcripts are acting and which pathways are involved. Future studies should broaden the scope of the viral transcripts used and the cell types. Cells with various specific parts of the innate immune system knocked out/down could be used to assess the effect on RNA induced IFN. There was also no direct relation between strength of IFN induction and other parameters such as genome GC content or the dinucleotide UA or CG ratios. There is much to learn about the interaction between viral RNA and cells, but the work presented in this chapter shows that that relationship is complex, and that GORS may be one part of the picture.

5.5 Future work

A study for which some preliminary work was carried out, was designed to quantify the dsRNA in cells during infection. A panel of predicted GORS and non-GORS viruses would be used to infect cells. At the same point on their respective growth curves the cells would fixed and, using the anti-dsRNA antibody, J2 (Scicons), fluorescence-activated cell sorting (FACS) would be used to quantify the level of dsRNA present. Viral copy number in cells infected at the same time would then be determined so that for each virus the level of dsRNA per virus copy number could be determined. The hypothesis would be that GORS virus infection would produce less detectable dsRNA per viral RNA copy than non-GORS viruses.

The panel of viruses chosen for the study were, the following GORS viruses: canine calicivirus (CaCV) (an isolate of which was kindly provided by Dr Erwin Duizer of the National Institute for Public Health and the Environment, Netherlands), ERAV and MNV; and the following non-GORS viruses: echovirus 11 (E11), Semliki Forest virus (provided by Prof John Fazakerley, University of Edinburgh) and Theilers murine encephalomyelitis virus. Each virus isolate was titrated by the end-point method and titres expressed as $TCID_{50}$ / ml. Seven wells of a 12-well plate were seeded with the following cell types for each virus:

CaCV - MDCK cells
ERAV - Vero cells
MNV - RAW 264.7 cells
E11 - RD cells
SFV - NIH3T3 cells
TMEV - NIH3T3 cells

Each well was infected with the appropriate virus and the cells were collected and frozen at 2, 4, 6 ,8 ,10, 12 and 24 hours post-infection, and stored at -80°C. Total cellular RNA was extracted by the RNEasy kit (Qiagen); the RNA was reverse transcribed into cDNA using the Reverse Transcription system (Promega).

Standard curves for quantitative real-time PCR were prepared as described previously. Briefly, RNA extracted from each virus was reverse transcribed and a PCR carried out. The PCR product was purified and the concentration determined by spectroscopy. Ten-fold dilution series were made according to copy number and used as the standard curves. Growth curves for each virus, based on intracellular viral copy number were made and are shown on figure 5.19 A-F. The plan was to infect two sets of cells; at the same point on each growth curve to measure the intracellular viral copy number in one group and to quantify the level of dsRNA in the other.

The anti-dsRNA, J2 (Scicons) has been used to demonstrate the presence of dsRNA in viral infection. dsRNA has been detected in infections with positive-sense, single-stranded RNA viruses, double stranded RNA viruses and DNA viruses, but not with negative strand RNA viruses (Weber *et al*., 2006). The plan was to use the J2 antibody in FACS analysis to quantify dsRNA. Some preliminary immunostaining of MDCK cells infected with CaCV and RD cells infected with E11 was carried out and multifocal, punctate bodies were seen (data not shown), but unfortunately the remainder of the planned experiment was not able to be carried out. This experiment, had it been successful, would not have relied upon IFN-β expression, which as has been discussed before, may vary considerably between cell lines; but would have attempted to quantify the level of PAMP production by GORS and non-GORS viruses.

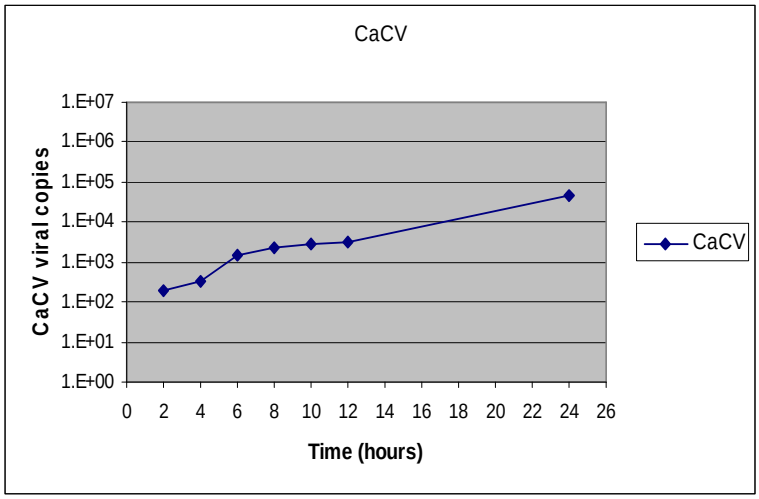


Figure 5.19 (A) 24 hour growth curve of canine calicivirus; measuring intracellular copy number by real-time PCR.

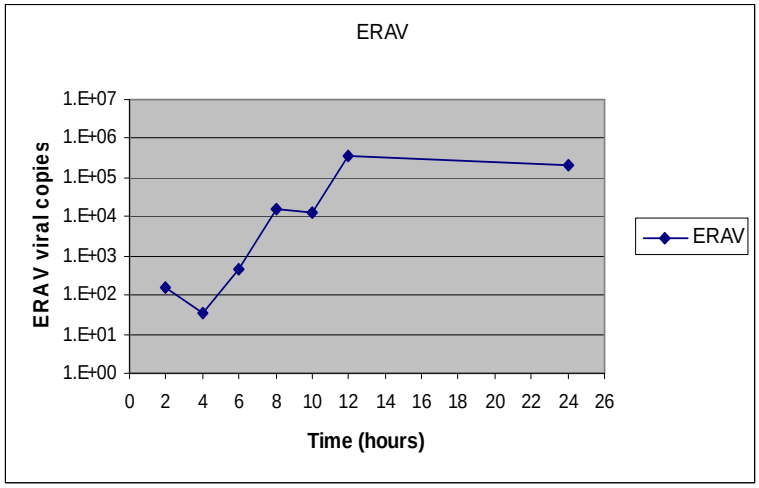


Figure 5.19 (B) 24 hour growth curve of equine rhinitis A virus; measuring intracellular copy number by real-time PCR.

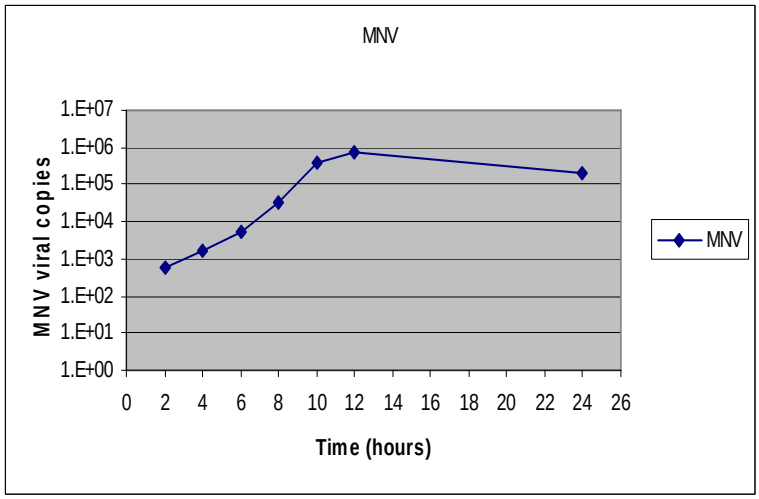


Figure 5.19 (C) 24 hour growth curve of murine norovirus; measuring intracellular copy number by real-time PCR.

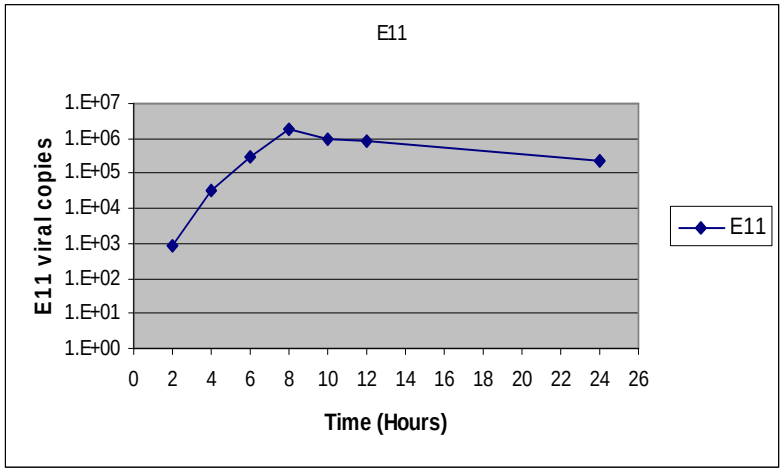


Figure 5.19 (D) 24 hour growth curve of echovirus 11; measuring intracellular copy number by real-time PCR.

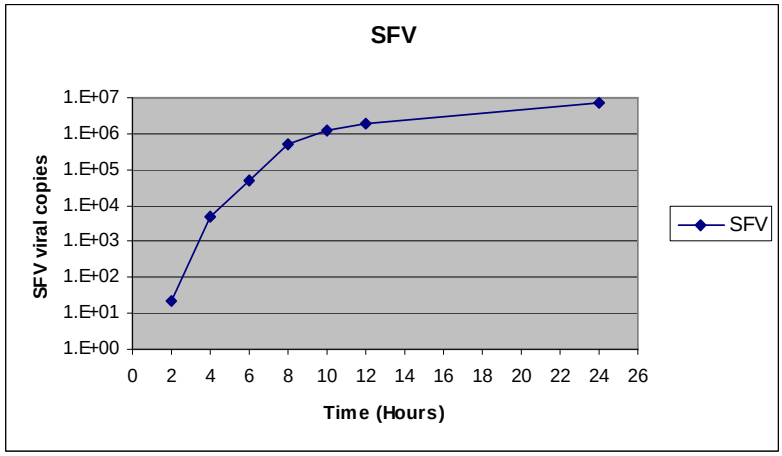


Figure 5.19 (E) 24 hour growth curve of Semliki Forest virus; measuring intracellular copy number by real-time PCR.

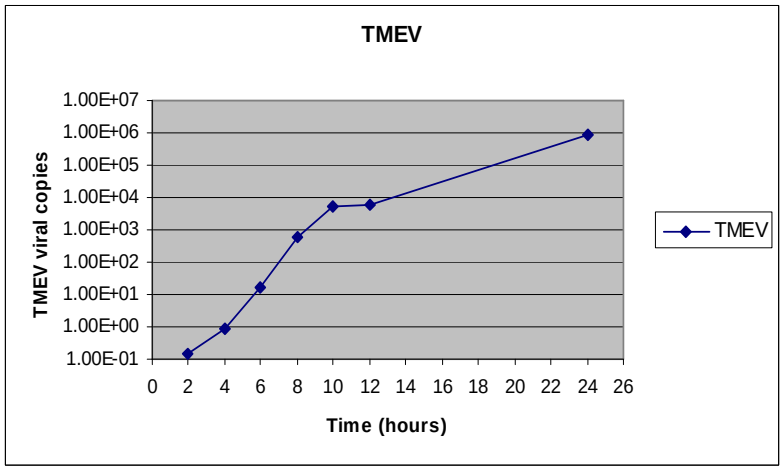


Figure 5.19 (F) 24 hour growth curve of Theilers murine encephalitis virus; measuring intracellular copy number by real-time PCR.

Chapter 6 Final discussion

Chapter 6 Final discussion

Genome-scale ordered RNA structure (GORS) was first described in 2004 by Simmonds *et al*. GORS was characterised by the presence of extensive predicted secondary structures along the lengths of the entire genomes of certain single-stranded, positive-sense RNA groups or genera. Viruses from the families *Picornaviridae* and *Flaviviridae* were included in the study, which analysed their RNA genomes by bioinformatic analysis, and it was shown that of the genera within those two families, some were predicted to be GORS and others non-GORS. It was considered that GORS could play not fundamental role in virus replication as replication strategies are generally conserved within virus families (Simmonds *et al*., 2004). Many RNA viruses have specific, conserved regions of secondary structure involved in replication e.g. IRES, CRE (Pelletier and Soenberg, 1988; Good fellow *et al*., 2000), but the presence of the extensive secondary structure in GORS appeared to be unrelated to these well characterised structures. The observation was also made that there was a relationship between viruses that contained GORS and the ability to establish persistent infections e.g. HCV, GBV-C, FMDV. It was speculated that GORS may be an evolutionarily maintained phenomenon as it confers an evolutionary advantage by contributing to viruses' ability to become persistent. The mechanism behind the contribution of GORS to persistence were unclear, but it was speculated that by adopting elaborate RNA secondary structures throughout the genome that the viruses would be able to evade recognition by cellular innate immunity (Simmonds *et al*., 2004). Subsequent work includes further bioinformatic predictions of GORS, including viruses from *Togaviridae* and *Caliciviridae*. It was shown that of the caliciviruses, the genus *Norovirus* contains genogroups which are predicted to be GORS (genogroup V containing MNV)) as well as non-GORS (other genogroups). As part of the same study, the physical nature of GORS was investigated by a probe hybridization accessibility assay of RNA transcripts. It was shown that the transcripts of predicted GORS viruses were markedly less accessible to the probes than predicted non-GORS viruses. Atomic force microscopy also revealed that GORS viral transcripts formed a tightly packed, condensed shape, whereas non-

GORS transcripts were pleomorphic and there were often protrusion of apparent single-stranded RNA (Davis *et al*., 2008)

This thesis present work carried out in the investigation of GORS and its role in viral persistence. There were two main paths of enquiry: the study of a GORS virus in cell culture and an investigation into the interaction between a panel of RNA transcripts of GORS and non-GORS viruses and the cellular mechanisms of RNA stability and innate immunity.

The aim of studying a virus whose genome contains GORS was to investigate the association between GORS and persistence in the natural host. The purpose was to identify viruses by PCR from animals with a naturally occurring persistent infection, which could then be analysed bioinformatically for secondary structure. Laboratory strains of viruses often become cell culture adapted and through mutations may lose features of wild-type viruses. The study of GORS viruses is somewhat complicated by the fact that some of them a not very convenient to work with the laboratory e.g. FMDV and HCV require category 3 containment facilities. ERAV is the only other member of the genus *Aphthovirus* apart from FMDV and is predicted to be a GORS virus. It causes a relatively mild upper respiratory tract infection and has been shown to be persistently shed for several weeks post-infection (McCollum and Timoney, 1992). We have shown the seroprevalence of ERAV in Scottish horse to be 56 %, which is comparable to similar studies in other countries (Kriegshauser *et al*., 2005; De Boer *et al*., 1979; Holmes *et al*., 1978; Steck *et al*., 1978). Unfortunately, it was not possible to isolate a wild type ERAV from an infected horse which could then have formed the basis of further work; and isolates kindly provided by the Animal Health Trust were the same strain as the published laboratory strain.

MNV, a norovirus of the family *Caliciviridae,* is the ideal virus to study GORS: the mean MFED of the first four published MNV sequences are 6.3 – 8.5 %; it establishes subclinical, persistent infections in immunocompetent mice (Hsu *et al*., 2006); uniquely among noroviruses there is a cell culture system available (Wobus *et al*., 2004) and there are infectious clones making it amenable to

reverse genetics (Chaudhry *et al*., 2007). This thesis has revealed a prevalence of MNV, by PCR screening of faecal samples, of 67 %; a similar value to other research facilities in the United States, Canada, Germany and Japan (Koto *et al*., 2009; Kitajima *et al*., 2009). Screening of faecal samples of wild mice revealed a surprisingly low prevalence, just one positive sample from a wood mouse (*Apodemus sylvaticus*) from a total of 56 samples (15 house mice (*Mus musculus*), 11 wood mice (*Apodemus sylvaticus*), 8 field voles (*Microtus agrestis*), and 12 bank voles (*Myodes glareolus*). A much wider prevalence study in wild rodents would provide a more accurate figure for the prevalence of MNV in wild rodents. As GORS has been associated with persistence, it would be expected that GORS viruses would be reasonably prevalent in wild animal populations, particularly if the viruses is able to replicate at a low level without causing a debilitating disease. A possible reason for the prevalence in laboratory mice being higher than that in wild mice is the absence of Mx in many laboratory mouse strains (Staeheli *et al*., 1988). Mx is one of the IFN induced genes that plays a role in the antiviral state, and the lack of Mx may make it easier for persistent infections to become established; wild animals will have fully competent immune systems. It is also true that MNV infection in immunocompetent mice is clinically silent, and its discovery by Karst *et al*. in 2003 was as the cause of widespread disease in a seriously immunocompromised, RAG/STAT$^{-/-}$ mouse. It is likely that MNV has existed in research facilities for some time before its discovery. The effect of the presence of MNV in laboratory mice in other studies is a current line of investigation, and has been shown to have little or no effect on experiments with murine cytomegalovirus (Doom *et al*., 2009) or on the adaptive immunity to vaccinia virus or influenza A virus (Hensley *et al*., 2009). Laboratory mice are also bred and live together in enclosed environments, allowing easier spread of pathogens; wild mice may not live in such density. It is interesting that the pet shop mouse samples were also positive for MNV as they are bred and raised in captivity and may live at a higher population density than wild mice. Phylogenetic analysis of the novel MNVs showed that the laboratory mouse and pet shop mouse MNVs were closely related to all previously published MNVs. The wood mouse MNV identified in this study may well be the first identified member of a new genetic cluster/genotype of norovirus genogroup V. Current

published norovirus classifications (Zheng *et al*., 2006) state that while the other norovirus genogroups contain different cluster, the MNVs made up a single cluster in genogroup V. This study demonstrates that there are likely to be more noroviruses in wild rodent species, and that maybe norovirus genogroup V has further undiscovered clusters; the data presented in this thesis provide the first evidence of that.

Bioinformatic analysis of the complete / almost complete genomes of these novel MNVs revealed them to be GORS viruses. The fact that the wood mouse virus can be classified as a member of genogroup V, and it genome also contains GORS agrees with previous observation on the distribution of GORS within the genus. The presence of GORS in the novel laboratory mouse MNVs also agrees with the description of MNV as a clinically silent, persistent infection in immunocompetent mice. The passage study of MNV-3 was designed to assess the impact of cell culture adaptation on GORS. The fact that, after 33 passages, there were so few mutations means that there was likely to be very little effect on the degree of secondary structure. The changes were non-synonymous so there must have been a degree of selection pressure during the passage series. It is unclear what those adaptations may have been. This study could be greatly expanded in the future study of the effect of GORS in viruses. Cell lines with various parts of the innate immune system knocked out could be generated, the hypothesis being that removing pressure from the immune system would make it less of an advantage for the virus to maintain that degree of genomic secondary structure. It is already known that mice the RAG/STAT$^{-/-}$ are susceptible to MNV-1, a challenge study of knockout mice deficient in various immune genes could provide an insight into the mechanism of persistence by tracking the course of infection. It is certainly easier to consider experiments of this kind in mice with MNV than in horses with ERAV, cattle with FMDV or chimpanzees with HCV for example.

The relationship between viruses and the immune system is particularly complex. Many viruses, over the course of their co-evolution with their hosts, have developed a panoply of means to evade aspects of the immune system in order to survive. Eukaryotic cells have likewise developed innate and adaptive

immune system in order to counteract the actions of invading pathogens. dsRNA is the most important pathogen associated molecular pattern (PAMP) produced during viral infections. It was speculated that the mechanism behind the ability of GORS viruses to establish persistence was as a result of the genome-scale structure to evade detection by cellular receptors by mimicking the structured elements of cellular RNAs. It must be pointed out that many viruses have evolved mechanisms to evade certain aspects of the innate or adaptive immune systems (see Chapter 1), but that not all of them are persistent; although in order to become persistent, viruses must prevent themselves from being destroyed by the host. The work presented in this thesis investigates the effect of transfecting RNA transcripts of GORS and non-GORS viruses into cells and measuring the rate of decay of those transcripts, and their capacity for IFN-β induction. This experimental design is necessarily artificial in that these transcripts from a panel of viruses, which naturally infect a broad range of cell types in different hosts, were transfected into two cell types, NIH3T3 and A549. The experiments were carried out in this way to eliminate others variables so that the only variable feature was the degree of secondary structure present in the transcripts. In natural infections, the importance of the presence of GORS in the viral genomes is likely to be most significant after uncoating; at this time the viral RNA is released into the cytoplasm where it goes on to interact with ribosomes to be translated. Production of the viral proteins and the establishment of replication complexes associated with cellular organelle membranes is likely to restrict the access of the cytoplasmic dsRNA receptors RIG-I and MDA-5. Other points in the viral life cycle that involve dsRNA include the production of replicatory intermediates and production of new progeny RNA genomes that go on to encapsidation, but again, there is likely to be shielding from cytoplasmic receptors during these processes. The transfection experiments presented, in which RNA is delivered to the cytoplasm, most resembles the situation after genome uncoating. The hypothesis of the study states that GORS genomes would induce IFN-β less strongly than non-GORS genomes; but the results are interesting in that while the GORS virus transcripts did indeed induce IFN weakly, the non-GORS transcripts were gave a variable response. It raises the question of the role of structure in the nature of the response, as well as what other factors may be involved in explaining the

differences observed. The plant virus PEMV2, a predicted GORS virus, failed to induce an IFN-β response. As a plant virus it will never have needed to evolve any other mechanism to avoid IFN as plant cells used RNAi as their mechanism of innate immunity (reviewed by Umbach and Cullen) – so the degree of secondary structure may have been the feature that prevented it from inducing a response in the mammalian cells, the RNA would certainly have been foreign to them. The failure of the SFV and RV transcripts is particularly surprising as i) they are both predicted non-GORS viruses and ii) they are both known to induce an IFN response in their natural infections and in cell culture (Fazakerley, 2002; Adamo *et al*., 2007). The reason for this is still unclear, but they both belong to the same family, *Togaviridae*, and there may be feature of togavirus RNA that has prevented these two transcripts from inducing IFN under these experimental conditions. While it was necessary for these studies to perform the experiments with all the viral transcripts simultaneously in the same cell line, it does not accurately reproduce the conditions during natural infection by each of these viruses in their own host cells. On the other hand, GORS is a feature that is observed across virus families and genera and is invariably associated with persistence, so the mechanism behind the role of GORS in persistence may common to all GORS viruses. Further work to investigate the interaction between GORS and non-GORS containing RNAs will be necessary to further elucidate the mechanisms behind the associated of GORS and persistence. The different reaction of NIH3T3 cells and A549 cells to the 4 kb transcripts also raises more questions. By making the 4 kb transcripts many variables could be removed from the experiment to focus the experiment uniquely on the effect of RNA secondary structure; but in nature the relationship between viral genomes and cells could be much more complicated and the presence of GORS may be only be part of a bigger picture. The longer transcripts are more likely to be representative of the situation during natural infection, and the division between those transcripts that induced IFN strongly and those that induced weakly or not at all was more marked in the long transcript experiments.

An experiment planned but unfortunately not completed, would have quantified intracytoplasmic dsRNA by means of FACS analysis using the anti-dsRNA

antibody, J2, during infection by a panel of GORS and non-GORS viruses. This could have given an indication as to the potential of each viruses to induce an IFN response; IFN could not be measure directly as many cell culture lines have deficiencies in parts of the innate immune system e.g. IFN deficiency in Vero cells.

This thesis set out to investigate the presence of GORS in positive-sense RNA viruses and its role in viral persistence. The investigation into MNV has confirmed that it is a common, subclinical infection of laboratory mice, is present in pet shop mice, and that a potentially new genetic MNV cluster exists in wild rodents; all of which have GORS; also that multiple passage in RAW 264.7 cells had little effect on the genome sequence. It has shown that GORS and non-GORS viral transcripts do not degrade at different rates after electroporation into cells. It has also shown, intriguingly, that transfection of a panel GORS and non-GORS viral RNA transcripts produce a marked variability in IFN-β induction, which while not confirming out original hypothesis, does not rule out a role for GORS in the evasion of innate immunity as a possible mechanism by which GORS viruses establish persistent infections.

Reference List

1. **Abendroth, A. and A. Arvin**. 2001. Immune evasion mechanisms of varicella-zoster virus. Arch. Virol. Suppl99-107.

2. **Accola, M. A., B. Huang, M. A. Al, and M. A. McNiven**. 2002. The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum. J. Biol. Chem. **277**:21829-21835.

3. **Adamo, M. P., M. Zapata, and T. K. Frey**. 2008. Analysis of gene expression in fetal and adult cells infected with rubella virus. Virology **370**:1-11.

4. **Adler, J. and R. Zickl**. 1969. Winter vomiting disease. J Infect Dis **119**:668-673.

5. **Aguilar, P. V., S. C. Weaver, and C. F. Basler**. 2007. Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. J. Virol. **81**:3866-3876.

6. **Alcami, A., J. A. Symons, and G. L. Smith**. 2000. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. J. Virol. **74**:11230-11239.

7. **Alexandersen, S., Z. Zhang, and A. I. Donaldson**. 2002. Aspects of the persistence of foot-and-mouth disease virus in animals--the carrier problem. Microbes. Infect. **4**:1099-1110.

8. **Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell**. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature **413**:732-738.

9. **Alff, P. J., I. N. Gavrilovskaya, E. Gorbunova, K. Endriss, Y. Chong, E. Geimonen, N. Sen, N. C. Reich, and E. R. Mackow**. 2006. The pathogenic NY-1 hantavirus G1 cytoplasmic tail inhibits RIG-I- and TBK-1-directed interferon responses. J. Virol. **80** :9676-9686.

10. **Ambert-Balay, K., F. Bon, G. F. Le, P. Pothier, and E. Kohli**. 2005. Characterization of new recombinant noroviruses. J. Clin. Microbiol. **43**:5179-5186.

11. **Ambros, V. and D. Baltimore**. 1980. Purification and properties of a HeLa cell enzyme able to remove the 5'-terminal protein from poliovirus RNA. J. Biol. Chem. **255**:6739-6744.

12. **Ando, T., J. S. Noel, and R. L. Fankhauser**. 2000. Genetic classification of "Norwalk-like viruses. J. Infect. Dis. **181 Suppl 2**:S336-S348.

13. **Andrejeva, J., K. S. Childs, D. F. Young, T. S. Carlos, N. Stock, S. Goodbourn, and R. E. Randall**. 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc. Natl. Acad. Sci. U. S. A **101**:17264-17269.

14. **Anton, L. C., U. Schubert, I. Bacik, M. F. Princiotta, P. A. Wearsch, J. Gibbs, P. M. Day, C. Realini, M. C. Rechsteiner, J. R. Bennink, and J. W. Yewdell**. 1999. Intracellular localization of proteasomal degradation of a viral antigen. J. Cell Biol. **146**:113-124.

15. **Asanaka, M., R. L. Atmar, V. Ruvolo, S. E. Crawford, F. H. Neill, and M. K. Estes**. 2005. Replication and packaging of Norwalk virus RNA in cultured mammalian cells. Proc. Natl. Acad. Sci. U. S. A **102**:10327-10332.

16. **Atmar, R. L. and M. K. Estes**. 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin. Microbiol. Rev. **14**:15-37.

17. **Baeuerle, P. A. and D. Baltimore**. 1988. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science **242**:540-546.

18. **Baeuerle, P. A. and D. Baltimore**. 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. Cell **53**:211-217.

19. **Balvay, L., R. S. Rifo, E. P. Ricci, D. Decimo, and T. Ohlmann**. 2009. Structural and functional diversity of viral IRESes. Biochim. Biophys. Acta.

20. **Banninger, G. and N. C. Reich**. 2004. STAT2 nuclear trafficking. J. Biol. Chem. **279**:39199-39206.

21. **Barnard, P. and N. A. McMillan**. 1999. The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. Virology **259**:305-313.

22. **Barry, G., L. Breakwell, R. Fragkoudis, G. ttarzadeh-Yazdi, J. Rodriguez-Andres, A. Kohl, and J. K. Fazakerley**. 2009. PKR acts early in infection to suppress Semliki Forest virus production and strongly enhances the type I interferon response. J. Gen. Virol. **90**:1382-1391.

23. **Bashkirov, V. I., H. Scherthan, J. A. Solinger, J. M. Buerstedde, and W. D. Heyer**. 1997. A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. J. Cell Biol. **136**:761-773.

24. **Bass, B. L. and H. Weintraub**. 1988. An unwinding activity that covalently modifies its double-stranded RNA substrate. Cell **55**:1089-1098.

25. **Bauhofer, O., A. Summerfield, K. C. McCullough, and N. Ruggli**. 2005. Role of double-stranded RNA and Npro of classical swine fever virus in the activation of monocyte-derived dendritic cells. Virology **343**:93-105.

26. **Beattie, E., J. Tartaglia, and E. Paoletti**. 1991. Vaccinia virus-encoded eIF-2 alpha homolog abrogates the antiviral effect of interferon. Virology **183**:419-422.

27. **Beck, E., S. Forss, K. Strebel, R. Cattaneo, and G. Feil**. 1983. Structure of the FMDV translation initiation site and of the structural proteins. Nucleic Acids Res. **11**:7873-7885.

28. **Becker, K. M., C. L. Moe, K. L. Southwick, and J. N. MacCormack**. 2000. Transmission of Norwalk virus during football game. N. Engl. J. Med. **343**:1223-1227.

29. **Becker, W., H. Heller, and P. Teufel**. 1974. [Equine rhinovirus infection]. Berl Munch. Tierarztl. Wochenschr. **87**:305-308.

30. **Belsham, G. J., G. M. McInerney, and N. Ross-Smith**. 2000. Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. J. Virol. **74**:272-280.

31. **Belsham, G. J.** 2009. Divergent picornavirus IRES elements. Virus Res. **139**:183-192.

32. **Bernardi, R. and P. P. Pandolfi**. 2003. Role of PML and the PML-nuclear body in the control of programmed cell death. Oncogene **22**:9048-9057.

33. **Bertolotti-Ciarlet, A., S. E. Crawford, A. M. Hutson, and M. K. Estes**. 2003. The 3' end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. J. Virol. **77**:11603-11615.

34. **Best, S. M., K. L. Morris, J. G. Shannon, S. J. Robertson, D. N. Mitzel, G. S. Park, E. Boer, J. B. Wolfinbarger, and M. E. Bloom**. 2005. Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist. J. Virol. **79**:12828-12839.

35. **Bhella, D., D. Gatherer, Y. Chaudhry, R. Pink, and I. G. Goodfellow**. 2008. Structural insights into calicivirus attachment and uncoating. J. Virol. **82**:8051-8058.

36. **Billerbeck, E., T. Bottler, and R. Thimme**. 2007. Regulatory T cells in viral hepatitis. World J. Gastroenterol. **13**:4858-4864.

37. **Bisbal, C. and R. H. Silverman**. 2007. Diverse functions of RNase L and implications in pathology. Biochimie **89**:789-798.

38. **Bischof, O., O. Kirsh, M. Pearson, K. Itahana, P. G. Pelicci, and A. Dejean**. 2002. Deconstructing PML-induced premature senescence. EMBO J. **21**:3358-3369.

39. **Black, T. L., G. N. Barber, and M. G. Katze**. 1993. Degradation of the interferon-induced 68,000-M(r) protein kinase by poliovirus requires RNA. J. Virol. **67**:791-800.

40. **Blacklow, N. R., G. Cukor, M. K. Bedigian, P. Echeverria, H. B. Greenberg, D. S. Schreiber, and J. S. Trier**. 1979. Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. J. Clin. Microbiol. **10**:903-909.

41. **Blakeney, S. J., A. Cahill, and P. A. Reilly**. 2003. Processing of Norwalk virus nonstructural proteins by a 3C-like cysteine proteinase. Virology **308**:216-224.

42. **Blindenbacher, A., F. H. Duong, L. Hunziker, S. T. Stutvoet, X. Wang, L. Terracciano, D. Moradpour, H. E. Blum, T. Alonzi, M. Tripodi, M. N. La, and M. H. Heim**. 2003. Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology **124**:1465-1475.

43. **Blomstrom, D. C., D. Fahey, R. Kutny, B. D. Korant, and Knight E Jr**. 1986. Molecular characterization of the interferon-induced 15-kDa protein. Molecular cloning and nucleotide and amino acid sequence. J. Biol. Chem. **261**:8811-8816.

44. **Bode, J. G., S. Ludwig, C. Ehrhardt, U. Albrecht, A. Erhardt, F. Schaper, P. C. Heinrich, and D. Haussinger**. 2003. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J. **17**:488-490.

45. **Boege, U., D. S. Ko, and D. G. Scraba**. 1986. Toward an in vitro system for picornavirus assembly: purification of mengovirus 14S capsid precursor particles. J. Virol. **57**:275-284.

46. **Bok, K., V. G. Prikhodko, K. Y. Green, and S. V. Sosnovtsev**. 2009. Apoptosis in murine norovirus-infected RAW264.7 cells is associated with downregulation of survivin. J. Virol. **83**:3647-3656.

47. **Bonilla, W. V., D. D. Pinschewer, P. Klenerman, V. Rousson, M. Gaboli, P. P. Pandolfi, R. M. Zinkernagel, M. S. Salvato, and H. Hengartner**. 2002. Effects of promyelocytic leukemia protein on virus-host balance. J. Virol. **76**:3810-3818.

48. **Bossert, B. and K. K. Conzelmann**. 2002. Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells. J. Virol. **76**:4287-4293.

49. **Bouloy, M., C. Janzen, P. Vialat, H. Khun, J. Pavlovic, M. Huerre, and O. Haller**. 2001. Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs. J. Virol. **75**:1371-1377.

50. **Boyman, O., J. F. Purton, C. D. Surh, and J. Sprent**. 2007. Cytokines and T-cell homeostasis. Curr. Opin. Immunol. **19**:320-326.

51. **Brass, V., R. Gosert, and D. Moradpour**. 2009. Investigation of the hepatitis C virus replication complex. Methods Mol. Biol. **510**:195-209.

52. **Breakwell, L., P. Dosenovic, G. B. Karlsson Hedestam, M. D'Amato, P. Liljestrom, J. Fazakerley, and G. M. McInerney**. 2007. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. J. Virol. **81**:8677-8684.

53. **Brion, A., C. Cateigne, M. Fontaine, M. P. Fontaine, and R. Moraillon**. 1966. [Equine influenza. Characterization of the influenza virus isolated in 1965]. Bull. Acad. Vet. Fr. **39**:155-163.

54. **Brown, E. A., H. Zhang, L. H. Ping, and S. M. Lemon**. 1992. Secondary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res. **20**:5041-5045.

55. **Brzozka, K., S. Finke, and K. K. Conzelmann**. 2006. Inhibition of interferon signaling by rabies virus phosphoprotein P: activation-dependent binding of STAT1 and STAT2. J. Virol. **80**:2675-2683.

56. **Burysek, L. and P. M. Pitha**. 2001. Latently expressed human herpesvirus 8-encoded interferon regulatory factor 2 inhibits double-stranded RNA-activated protein kinase. J. Virol. **75**:2345-2352.

57. **Camus-Bouclainville, C., L. Fiette, S. Bouchiha, B. Pignolet, D. Counor, C. Filipe, J. Gelfi, and F. Messud-Petit** . 2004. A virulence factor of myxoma virus colocalizes with NF-kappaB in the nucleus and interferes with inflammation. J. Virol. **78**:2510-2516.

58. **Cao, Y., Z. Lu, J. Sun, X. Bai, P. Sun, H. Bao, Y. Chen, J. Guo, D. Li, X. Liu, and Z. Liu**. 2009. Synthesis of empty capsid-like particles of Asia I foot-and-mouth disease virus in insect cells and their immunogenicity in guinea pigs. Vet. Microbiol. **137**:10-17.

59. **Cardenas, W. B., Y. M. Loo, M. Gale, Jr., A. L. Hartman, C. R. Kimberlin, L. Martinez-Sobrido, E. O. Saphire, and C. F. Basler**. 2006. Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. J. Virol. **80**:5168-5178.

60. **Carrillo, E. C., C. Giachetti, and R. H. Campos**. 1984. Effect of lysosomotropic agents on the foot-and-mouth disease virus replication. Virology **135**:542-545.

61. **Carrillo, E. C., C. Giachetti, and R. Campos**. 1985. Early steps in FMDV replication: further analysis on the effects of chloroquine. Virology **147**:118-125.

62. **Caspar, D. L. and A. Klug**. 1962. Physical principles in the construction of regular viruses. Cold Spring Harb. Symp. Quant. Biol. **27**:1-24.

63. **Castelli, J., K. A. Wood, and R. J. Youle**. 1998. The 2-5A system in viral infection and apoptosis. Biomed. Pharmacother. **52**:386-390.

64. **Castelli, J. C., B. A. Hassel, A. Maran, J. Paranjape, J. A. Hewitt, X. L. Li, Y. T. Hsu, R. H. Silverman, and R. J. Youle**. 1998. The role of 2'-5' oligoadenylate-activated ribonuclease L in apoptosis. Cell Death. Differ. **5**:313-320.

65. **Catic, A., E. Fiebiger, G. A. Korbel, D. Blom, P. J. Galardy, and H. L. Ploegh**. 2007. Screen for ISG15-crossreactive deubiquitinases. PLoS. One. **2**:e679.

66. **Cattaneo, R.** 1994. Biased (A-->I) hypermutation of animal RNA virus genomes. Curr. Opin. Genet. Dev. **4**:895-900.

67. **Chachu, K. A., A. D. LoBue, D. W. Strong, R. S. Baric, and H. W. Virgin**. 2008. Immune mechanisms responsible for vaccination against and clearance of mucosal and lymphatic norovirus infection. PLoS. Pathog. **4**:e1000236.

68. **Chang, H. W., J. C. Watson, and B. L. Jacobs**. 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. Proc. Natl. Acad. Sci. U. S. A **89**:4825-4829.

69. **Changotra, H., Y. Jia, T. N. Moore, G. Liu, S. M. Kahan, S. V. Sosnovtsev, and S. M. Karst**. 2009. Type I and type II interferons inhibit the translation of murine norovirus proteins. J. Virol. **83**:5683-5692.

70. **Chaudhry, Y., A. Nayak, M. E. Bordeleau, J. Tanaka, J. Pelletier, G. J. Belsham, L. O. Roberts, and I. G. Goodfellow**. 2006. Caliciviruses differ in their functional requirements for eIF4F components. J. Biol. Chem. **281**:25315-25325.

71. **Chaudhry, Y., M. A. Skinner, and I. G. Goodfellow**. 2007. Recovery of genetically defined murine norovirus in tissue culture by using a fowlpox virus expressing T7 RNA polymerase. J. Gen. Virol. **88**:2091-2100.

72. **Chee, A. V. and B. Roizman**. 2004. Herpes simplex virus 1 gene products occlude the interferon signaling pathway at multiple sites. J. Virol. **78**:4185-4196.

73. **Chelbi-Alix, M. K., L. Pelicano, F. Quignon, M. H. Koken, L. Venturini, M. Stadler, J. Pavlovic, L. Degos, and T. H. De**. 1995. Induction of the PML protein by interferons in normal and APL cells. Leukemia **9**:2027-2033.

74. **Chelbi-Alix, M. K., F. Quignon, L. Pelicano, M. H. Koken, and T. H. De**. 1998. Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. J. Virol. **72**:1043-1051.

75. **Chen, R., J. D. Neill, M. K. Estes, and B. V. Prasad**. 2006. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. Proc. Natl. Acad. Sci. U. S. A **103**:8048-8053.

76. **Chen, Z. J.** 2005. Ubiquitin signalling in the NF-kappaB pathway. Nat. Cell Biol. **7**:758-765.

77. **Child, S. J., L. K. Hanson, C. E. Brown, D. M. Janzen, and A. P. Geballe**. 2006. Double-stranded RNA binding by a heterodimeric complex of murine cytomegalovirus m142 and m143 proteins. J. Virol. **80**:10173-10180.

78. **Childs, K., N. Stock, C. Ross, J. Andrejeva, L. Hilton, M. Skinner, R. Randall, and S. Goodbourn**. 2007. mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. Virology **359**:190-200.

79. **Childs, K. S., J. Andrejeva, R. E. Randall, and S. Goodbourn**. 2009. Mechanism of mda-5 Inhibition by paramyxovirus V proteins. J. Virol. **83**:1465-1473.

80. **Chin, K. C. and P. Cresswell**. 2001. Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc. Natl. Acad. Sci. U. S. A **98**:15125-15130.

81. **Chin, Y. E., M. Kitagawa, W. C. Su, Z. H. You, Y. Iwamoto, and X. Y. Fu**. 1996. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science **272**:719-722.

82. **Chin, Y. E., M. Kitagawa, K. Kuida, R. A. Flavell, and X. Y. Fu**. 1997. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Mol. Cell Biol. **17**:5328-5337.

83. **Chou, J., J. J. Chen, M. Gross, and B. Roizman**. 1995. Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 alpha and premature shutoff of protein synthesis after infection with gamma 134.5- mutants of herpes simplex virus 1. Proc. Natl. Acad. Sci. U. S. A **92**:10516-10520.

84. **Chow, M., J. F. Newman, D. Filman, J. M. Hogle, D. J. Rowlands, and F. Brown**. 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature **327**:482-486.

85. **Christen, V., F. Duong, C. Bernsmeier, D. Sun, M. Nassal, and M. H. Heim**. 2007. Inhibition of alpha interferon signaling by hepatitis B virus. J. Virol. **81**:159-165.

86. **Clark, M. E., P. M. Lieberman, A. J. Berk, and A. Dasgupta**. 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. Mol. Cell Biol. **13**:1232-1237.

87. **Clarke, B. E., D. V. Sangar, J. N. Burroughs, S. E. Newton, A. R. Carroll, and D. J. Rowlands**. 1985. Two initiation sites for foot-and-mouth disease virus polyprotein in vivo. J. Gen. Virol. **66 (Pt 12)**:2615-2626.

88. **Clemens, M. J. and B. R. Williams** . 1978. Inhibition of cell-free protein synthesis by pppA2'p5'A2'p5'A: a novel oligonucleotide synthesized by interferon-treated L cell extracts. Cell **13**:565-572.

89. **Clemens, M. J.** 1997. PKR--a protein kinase regulated by double-stranded RNA. Int. J. Biochem. Cell Biol. **29**:945-949.

90. **Clemens, M. J.** 2005. Translational control in virus-infected cells: models for cellular stress responses. Semin. Cell Dev. Biol. **16**:13-20.

91. **Colamonici, O. R., P. Domanski, S. M. Sweitzer, A. Larner, and R. M. Buller**. 1995. Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling. J. Biol. Chem. **270**:15974-15978.

92. **Comoli, P., H. H. Hirsch, and F. Ginevri**. 2008. Cellular immune responses to BK virus. Curr. Opin. Organ Transplant. **13**:569-574.

93. **Cougot, N., S. Babajko, and B. Seraphin**. 2004. Cytoplasmic foci are sites of mRNA decay in human cells. J. Cell Biol. **165**:31-40.

94. **Cuddihy, A. R., A. H. Wong, N. W. Tam, S. Li, and A. E. Koromilas**. 1999. The double-stranded RNA activated protein kinase PKR physically associates with the tumor suppressor p53 protein and phosphorylates human p53 on serine 392 in vitro. Oncogene **18**:2690-2702.

95. **Cui, S., K. Eisenacher, A. Kirchhofer, K. Brzozka, A. Lammens, K. Lammens, T. Fujita, K. K. Conzelmann, A. Krug, and K. P. Hopfner**. 2008. The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. Mol. Cell **29**:169-179.

96. **Cusson-Hermance, N., S. Khurana, T. H. Lee, K. A. Fitzgerald, and M. A. Kelliher**. 2005. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J. Biol. Chem. **280**:36560-36566.

97. **D'Cunha, J., S. Ramanujam, R. J. Wagner, P. L. Witt, Knight E Jr, and E. C. Borden**. 1996. In vitro and in vivo secretion of human ISG15, an IFN-induced immunomodulatory cytokine. J. Immunol. **157**:4100-4108.

98. **Dai, C. and S. B. Krantz**. 1999. Interferon gamma induces upregulation and activation of caspases 1, 3, and 8 to produce apoptosis in human erythroid progenitor cells. Blood **93**:3309-3316.

99. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark**. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264**:1415-1421.

100. **Darnell, J. E., Jr.** 1997. STATs and gene regulation. Science **277**:1630-1635.

101. **Daughenbaugh, K. F., C. S. Fraser, J. W. Hershey, and M. E. Hardy**. 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. EMBO J. **22**:2852-2859.

102. **Daughenbaugh, K. F., C. E. Wobus, and M. E. Hardy**. 2006. VPg of murine norovirus binds translation initiation factors in infected cells. Virol. J. **3**:33.

103. **Davis, M., S. M. Sagan, J. P. Pezacki, D. J. Evans, and P. Simmonds**. 2008. Bioinformatic and physical characterizations of genome-scale ordered RNA structure in mammalian RNA viruses. J. Virol. **82**:11824-11836.

104. **Dawson, M. J. and J. A. Trapani**. 1996. HIN-200: a novel family of IFN-inducible nuclear proteins expressed in leukocytes. J. Leukoc. Biol. **60**:310-316.

105. **de Boer, G. F., A. D. Osterhaus, J. T. van Oirschot, and R. Wemmenhove**. 1979. Prevalence of antibodies to equine viruses in the Netherlands. Tijdschr. Diergeneeskd. **104** :suppl-74.

106. **de Weerd, N. A., S. A. Samarajiwa, and P. J. Hertzog**. 2007. Type I interferon receptors: biochemistry and biological functions. J. Biol. Chem. **282**:20053-20057.

107. **de, Bouteiller. O., E. Merck, U. A. Hasan, S. Hubac, B. Benguigui, G. Trinchieri, E. E. Bates, and C. Caux**. 2005. Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J. Biol. Chem. **280**:38133-38145.

108. **de, Haro. C., R. Mendez, and J. Santoyo**. 1996. The eIF-2alpha kinases and the control of protein synthesis. FASEB J. **10**:1378-1387.

109. **Delhaye, S., P. van, V, and T. Michiels**. 2004. The leader protein of Theiler's virus interferes with nucleocytoplasmic trafficking of cellular proteins. J. Virol. **78**:4357-4362.

110. **Dellaire, G. and D. P. Bazett-Jones**. 2004. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. Bioessays **26**:963-977.

111. **Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, and Z. J. Chen**. 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell **103**:351-361.

112. **Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman**. 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Natl. Acad. Sci. U. S. A **95**:15623-15628.

113. **Devaney, M. A., V. N. Vakharia, R. E. Lloyd, E. Ehrenfeld, and M. J. Grubman**. 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. J. Virol. **62**:4407-4409.

114. **Diao, F., S. Li, Y. Tian, M. Zhang, L. G. Xu, Y. Zhang, R. P. Wang, D. Chen, Z. Zhai, B. Zhong, P. Tien, and H. B. Shu**. 2007. Negative regulation of MDA5 but not RIG-I-mediated innate antiviral signalling by the dihydroxyacetone kinase. Proc. Natl. Acad. Sci. U. S. A **104**:11706-11711.

115. **DiPerna, G., J. Stack, A. G. Bowie, A. Boyd, G. Kotwal, Z. Zhang, S. Arvikar, E. Latz, K. A. Fitzgerald, and W. L. Marshall**. 2004. Poxvirus protein N1L targets the I-kappaB kinase complex, inhibits signaling to NF-kappaB by the tumor necrosis factor superfamily of receptors, and inhibits NF-kappaB and IRF3 signaling by toll-like receptors. J. Biol. Chem. **279**:36570-36578.

116. **Ditchfield, J. and L. W. MacPherson**. 1965. THE PROPERTIES AND CLASSIFICATION OF TWO NEW RHINOVIRUSES RECOVERED FROM HORSES IN TORONTO, CANADA. Cornell Vet. **55**:181-189.

117. **Dolin, R., A. G. Levy, R. G. Wyatt, T. S. Thornhill, and J. D. Gardner**. 1975. Viral gastroenteritis induced by the Hawaii agent. Jejunal histopathology and serologic response. Am. J. Med. **59**:761-768.

118. **Dolin, R., R. C. Reichman, and A. S. Fauci**. 1976. Lymphocyte populations in acute viral gastroenteritis. Infect. Immun. **14**:422-428.

119. **Dong, B. and R. H. Silverman**. 1995. 2-5A-dependent RNase molecules dimerize during activation by 2-5A. J. Biol. Chem. **270**:4133-4137.

120. **Dong, B. and R. H. Silverman**. 1997. A bipartite model of 2-5A-dependent RNase L. J. Biol. Chem. **272**:22236-22242.

121. **Dong, B., M. Niwa, P. Walter, and R. H. Silverman**. 2001. Basis for regulated RNA cleavage by functional analysis of RNase L and Ire1p. RNA. **7**:361-373.

122. **Doom, C. M., H. M. Turula, and A. B. Hill**. 2009. Investigation of the impact of the common animal facility contaminant murine norovirus on experimental murine cytomegalovirus infection. Virology **392**:153-161.

123. **Doucas, V., A. M. Ishov, A. Romo, H. Juguilon, M. D. Weitzman, R. M. Evans, and G. G. Maul**. 1996. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. Genes Dev. **10**:196-207.

124. **Dragan, A. I., V. V. Hargreaves, E. N. Makeyeva, and P. L. Privalov**. 2007. Mechanisms of activation of interferon regulator factor 3: the role of C-terminal domain phosphorylation in IRF-3 dimerization and DNA binding. Nucleic Acids Res. **35**:3525-3534.

125. **Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. Koopmans, and M. K. Estes**. 2004. Laboratory efforts to cultivate noroviruses. J. Gen. Virol. **85**:79-87.

126. **Duizer, E., P. Bijkerk, B. Rockx, G. A. De, F. Twisk, and M. Koopmans**. 2004. Inactivation of caliciviruses. Appl. Environ. Microbiol. **70**:4538-4543.

127. **Duong, F. H., M. Filipowicz, M. Tripodi, M. N. La, and M. H. Heim**. 2004. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. Gastroenterology **126**:263-277.

128. **Ea, C. K., L. Deng, Z. P. Xia, G. Pineda, and Z. J. Chen**. 2006. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell **22**:245-257.

129. **Edelmann, K. H., S. Richardson-Burns, L. Alexopoulou, K. L. Tyler, R. A. Flavell, and M. B. Oldstone**. 2004. Does Toll-like receptor 3 play a biological role in virus infections? Virology **322**:231-238.

130. **Endo-Munoz, L., T. Warby, D. Harrich, and N. A. McMillan**. 2005. Phosphorylation of HIV Tat by PKR increases interaction with TAR RNA and enhances transcription. Virol. J. **2**:17.

131. **Erlandsson, L., R. Blumenthal, M. L. Eloranta, H. Engel, G. Alm, S. Weiss, and T. Leanderson**. 1998. Interferon-beta is required for interferon-alpha production in mouse fibroblasts. Curr. Biol. **8**:223-226.

132. **Escarmis, C., J. Dopazo, M. Davila, E. L. Palma, and E. Domingo**. 1995. Large deletions in the 5'-untranslated region of foot-and-mouth disease virus of serotype C. Virus Res. **35**:155-167.

133. **Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. Hershey**. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem. **257**:14806-14810.

134. **Eulalio, A., I. Behm-Ansmant, D. Schweizer, and E. Izaurralde**. 2007. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol. Cell Biol. **27**:3970-3981.

135. **Farci, P. and R. H. Purcell**. 2000. Clinical significance of hepatitis C virus genotypes and quasispecies. Semin. Liver Dis. **20**:103-126.

136. **Farkas, T., K. Sestak, C. Wei, and X. Jiang**. 2008. Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae. J. Virol. **82**:5408-5416.

137. **Fazakerley, J. K.** 2002. Pathogenesis of Semliki Forest virus encephalitis. J. Neurovirol. **8 Suppl 2**:66-74.

138. **Fernandez, M., J. A. Quiroga, and V. Carreno**. 2003. Hepatitis B virus downregulates the human interferon-inducible MxA promoter through direct interaction of precore/core proteins. J. Gen. Virol. **84**:2073-2082.

139. **Ferraiuolo, M. A., S. Basak, J. Dostie, E. L. Murray, D. R. Schoenberg, and N. Sonenberg**. 2005. A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. J. Cell Biol. **170**:913-924.

140. **Ferran, M. C. and J. M. Lucas-Lenard**. 1997. The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. J. Virol. **71**:371-377.

141. **Ferrantini, M., I. Capone, and F. Belardelli**. 2007. Interferon-alpha and cancer: mechanisms of action and new perspectives of clinical use. Biochimie **89**:884-893.

142. **Fessler, S. P., Y. R. Chin, and M. S. Horwitz**. 2004. Inhibition of tumor necrosis factor (TNF) signal transduction by the adenovirus group C RID complex involves downregulation of surface levels of TNF receptor 1. J. Virol. **78**:13113-13121.

143. **Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman**. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc. Natl. Acad. Sci. U. S. A **58**:1004-1010.

144. **Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello**. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391**:806-811.

145. **Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis**. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. **4**:491-496.

146. **Flammini, C. F. and G. Allegri**. 1970. Rhinovirus strain as a possible cause of equine respiratory infection. Arch. Vet. Ital. **21**:309-316.

147. **Flanegan, J. B., R. F. Petterson, V. Ambros, N. J. Hewlett, and D. Baltimore**. 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5'-terminus of virion and replicative intermediate RNAs of poliovirus. Proc. Natl. Acad. Sci. U. S. A **74**:961-965.

148. **Flodstrom-Tullberg, M., M. Hultcrantz, A. Stotland, A. Maday, D. Tsai, C. Fine, B. Williams, R. Silverman, and N. Sarvetnick**. 2005. RNase L and double-stranded RNA-dependent protein kinase exert complementary roles in islet cell defense during coxsackievirus infection. J. Immunol. **174**:1171-1177.

149. **Floyd-Smith, G., E. Slattery, and P. Lengyel**. 1981. Interferon action: RNA cleavage pattern of a (2'-5')oligoadenylate--dependent endonuclease. Science **212**:1030-1032.

150. **Fontana, J., N. Lopez-Montero, R. M. Elliott, J. J. Fernandez, and C. Risco**. 2008. The unique architecture of Bunyamwera virus factories around the Golgi complex. Cell Microbiol. **10**:2012-2028.

151. **Forss, S. and H. Schaller**. 1982. A tandem repeat gene in a picornavirus. Nucleic Acids Res. **10**:6441-6450.

152. **Fortes, P., A. Beloso, and J. Ortin**. 1994. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. EMBO J. **13**:704-712.

153. **Fox, G., N. R. Parry, P. V. Barnett, B. McGinn, D. J. Rowlands, and F. Brown**. 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J. Gen. Virol. **70 (Pt 3)**:625-637.

154. **Foy, E., K. Li, R. Sumpter, Jr., Y. M. Loo, C. L. Johnson, C. Wang, P. M. Fish, M. Yoneyama, T. Fujita, S. M. Lemon, and M. Gale, Jr.** 2005. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc. Natl. Acad. Sci. U. S. A **102**:2986-2991.

155. **Fragkoudis, R., L. Breakwell, C. McKimmie, A. Boyd, G. Barry, A. Kohl, A. Merits, and J. K. Fazakerley**. 2007. The type I interferon system protects mice from Semliki Forest virus by preventing widespread virus dissemination in extraneural tissues, but does not mediate the restricted replication of avirulent virus in central nervous system neurons. J. Gen. Virol. **88**:3373-3384.

156. **Fragkoudis, R., Y. Chi, R. W. Siu, G. Barry, G. ttarzadeh-Yazdi, A. Merits, A. A. Nash, J. K. Fazakerley, and A. Kohl**. 2008. Semliki Forest virus strongly reduces mosquito host defence signaling. Insect Mol. Biol. **17**:647-656.

157. **Frahm, T., H. Hauser, and M. Koster**. 2006. IFN-type-I-mediated signaling is regulated by modulation of STAT2 nuclear export. J. Cell Sci. **119**:1092-1104.

158. **Fresco, J. R., B. M. Alberts, and P. Doty**. 1960. Some molecular details of the secondary structure of ribonucleic acid. Nature **188**:98-101.

159. **Friedman, J. M. and M. S. Horwitz**. 2002. Inhibition of tumor necrosis factor alpha-induced NF-kappa B activation by the adenovirus E3-10.4/14.5K complex. J. Virol. **76**:5515-5521.

160. **Friedman, R. M.** 1967. Interferon binding: the first step in establishment of antiviral activity. Science **156**:1760-1761.

161. **Frieman, M., B. Yount, M. Heise, S. A. Kopecky-Bromberg, P. Palese, and R. S. Baric**. 2007. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. J. Virol. **81**:9812-9824.

162. **Fry, E. E., S. M. Lea, T. Jackson, J. W. Newman, F. M. Ellard, W. E. Blakemore, R. bu-Ghazaleh, A. Samuel, A. M. King, and D. I. Stuart**. 1999. The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. EMBO J. **18** :543-554.

163. **Fu, X. Y., C. Schindler, T. Improta, R. Aebersold, and J. E. Darnell, Jr.** 1992. The proteins of ISGF-3, the interferon alpha-induced transcriptional

activator, define a gene family involved in signal transduction. Proc. Natl. Acad. Sci. U. S. A **89**:7840-7843.

164. **Fukumi, H., R. Nakaya, S. Hatta, H. Noriki, H. Yunoki, K. Kagi, T. Saito, K. Uchiyama, K. Kobari, and R. Nakanishi**. 1957. An indication as to identity between the infectious diarrhea in Japan and the afebrile infectious nonbacterial gastroenteritis by human volunteer experiments. Jpn. J. Med. Sci. Biol. **10**:1-17.

165. **Fukushi, S., K. Katayama, C. Kurihara, N. Ishiyama, F. B. Hoshino, T. Ando, and A. Oya**. 1994. Complete 5' noncoding region is necessary for the efficient internal initiation of hepatitis C virus RNA. Biochem. Biophys. Res. Commun. **199**:425-432.

166. **Gack, M. U., Y. C. Shin, C. H. Joo, T. Urano, C. Liang, L. Sun, O. Takeuchi, S. Akira, Z. Chen, S. Inoue, and J. U. Jung**. 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature **446**:916-920.

167. **Gaddy, D. F. and D. S. Lyles**. 2007. Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx. J. Virol. **81**:2792-2804.

168. **Gale, M., Jr. and E. M. Foy**. 2005. Evasion of intracellular host defence by hepatitis C virus. Nature **436**:939-945.

169. **Garcia, M. A., J. Gil, I. Ventoso, S. Guerra, E. Domingo, C. Rivas, and M. Esteban**. 2006. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol. Mol. Biol. Rev. **70**:1032-1060.

170. **Garcin, D., J. B. Marq, L. Strahle, M. P. le, and D. Kolakofsky**. 2002. All four Sendai Virus C proteins bind Stat1, but only the larger forms also induce its mono-ubiquitination and degradation. Virology **295**:256-265.

171. **Garneau, N. L., J. Wilusz, and C. J. Wilusz**. 2007. The highways and byways of mRNA decay. Nat. Rev. Mol. Cell Biol. **8**:113-126.

172. **Gatignol, A., A. Buckler-White, B. Berkhout, and K. T. Jeang**. 1991. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science **251**:1597-1600.

173. **George, C. X. and C. E. Samuel**. 1999. Human RNA-specific adenosine deaminase ADAR1 transcripts possess alternative exon 1 structures that initiate from different promoters, one constitutively active and the other interferon inducible. Proc. Natl. Acad. Sci. U. S. A **96**:4621-4626.

174. **Giannakopoulos, N. V., J. K. Luo, V. Papov, W. Zou, D. J. Lenschow, B. S. Jacobs, E. C. Borden, J. Li, H. W. Virgin, and D. E. Zhang**. 2005. Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. Biochem. Biophys. Res. Commun. **336**:496-506.

175. **Gil, J., J. Alcami, and M. Esteban**. 1999. Induction of apoptosis by double-stranded-RNA-dependent protein kinase (PKR) involves the alpha subunit of eukaryotic translation initiation factor 2 and NF-kappaB. Mol. Cell Biol. **19**:4653-4663.

176. **Girard, M., D. Baltimore, and J. Darnell**. 1967. The poliovirus replication complex: Sites for synthesis of poliovirus RNA. J. Mol. Biol. **24**:59-74.

177. **Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna**. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. U. S. A **103**:8459-8464.

178. **Glass, P. J., L. J. White, J. M. Ball, I. Leparc-Goffart, M. E. Hardy, and M. K. Estes**. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J. Virol. **74**:6581-6591.

179. **Goodfellow, I., Y. Chaudhry, I. Gioldasi, A. Gerondopoulos, A. Natoni, L. Labrie, J. F. Laliberte, and L. Roberts**. 2005. Calicivirus translation initiation requires an interaction between VPg and eIF 4 E. EMBO Rep. **6**:968-972.

180. **Goto, K., N. Hayashimoto, M. Yasuda, T. Ishida, S. Kameda, A. Takakura, and T. Itoh**. 2009. Molecular detection of murine norovirus from experimentally and spontaneously infected mice. Exp. Anim **58**:135-140.

181. **Graff, J. W., J. Ewen, K. Ettayebi, and M. E. Hardy**. 2007. Zinc-binding domain of rotavirus NSP1 is required for proteasome-dependent degradation of IRF3 and autoregulatory NSP1 stability. J. Gen. Virol. **88**:613-620.

182. **Gray, J. J., X. Jiang, P. Morgan-Capner, U. Desselberger, and M. K. Estes**. 1993. Prevalence of antibodies to Norwalk virus in England: detection by enzyme-linked immunosorbent assay using baculovirus-expressed Norwalk virus capsid antigen. J. Clin. Microbiol. **31**:1022-1025.

183. **Green**. 2007. Caliciviridae, p. 949-979. *In* D. Knipe and P. Howley (eds.), Fields Virology.

184. **Green, K. Y., T. Ando, M. S. Balayan, T. Berke, I. N. Clarke, M. K. Estes, D. O. Matson, S. Nakata, J. D. Neill, M. J. Studdert, and H. J. Thiel**. 2000. Taxonomy of the caliciviruses. J. Infect. Dis. **181 Suppl 2**:S322-S330.

185. **Greenberg, H. B. and A. Z. Kapikian**. 1978. Detection of Norwalk agent antibody and antigen by solid-phase radioimmunoassay and immune adherence hemagglutination assay. J. Am. Vet. Med. Assoc. **173**:620-623.

186. **Greenlund, A. C., M. A. Farrar, B. L. Viviano, and R. D. Schreiber**. 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J. **13**:1591-1600.

187. **Gruber, A. R., R. Neubock, I. L. Hofacker, and S. Washietl**. 2007. The RNAz web server: prediction of thermodynamically stable and evolutionarily conserved RNA structures. Nucleic Acids Res. **35**:W335-W338.

188. **Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena**. 2002. Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol. **20**:621-667.

189. **Gunn, R. A., W. A. Terranova, H. B. Greenberg, J. Yashuk, G. W. Gary, J. G. Wells, P. R. Taylor, and R. A. Feldman**. 1980. Norwalk virus gastroenteritis aboard a cruise ship: an outbreak on five consecutive cruises. Am. J. Epidemiol. **112**:820-827.

190. **Gunther, H. and P. Otto**. 1987. [Diarrhea in young calves. 7. "Zackenvirus" (Jena agent 117/80)--a new diarrhea pathogen in calves]. Arch. Exp. Veterinarmed. **41**:934-938.

191. **Guo, J., D. J. Hui, W. C. Merrick, and G. C. Sen**. 2000. A new pathway of translational regulation mediated by eukaryotic initiation factor 3. EMBO J. **19**:6891-6899.

192. **Guo, Z., L. M. Chen, H. Zeng, J. A. Gomez, J. Plowden, T. Fujita, J. M. Katz, R. O. Donis, and S. Sambhara**. 2007. NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. Am. J. Respir. Cell Mol. Biol. **36**:263-269.

193. **Gutierrez-Escolano, A. L., M. Vazquez-Ochoa, J. Escobar-Herrera, and J. Hernandez-Acosta**. 2003. La, PTB, and PAB proteins bind to the 3(') untranslated region of Norwalk virus genomic RNA. Biochem. Biophys. Res. Commun. **311**:759-766.

194. **Haasnoot, P. C., R. C. Olsthoorn, and J. F. Bol**. 2002. The Brome mosaic virus subgenomic promoter hairpin is structurally similar to the iron-responsive element and functionally equivalent to the minus-strand core promoter stem-loop C. RNA. **8**:110-122.

195. **Hacker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L. C. Hsu, G. G. Wang, M. P. Kamps, E. Raz, H. Wagner, G. Hacker, M. Mann, and M. Karin**. 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature **439**:204-207.

196. **Hagmaier, K., H. R. Gelderblom, and G. Kochs**. 2004. Functional comparison of the two gene products of Thogoto virus segment 6. J. Gen. Virol. **85**:3699-3708.

197. **Hagmaier, K., N. Stock, B. Precious, K. Childs, L. F. Wang, S. Goodbourn, and R. E. Randall**. 2007. Mapuera virus, a rubulavirus that inhibits interferon signalling in a wide variety of mammalian cells without degrading STATs. J. Gen. Virol. **88**:956-966.

198. **Hahm, B., M. J. Trifilo, E. I. Zuniga, and M. B. Oldstone**. 2005. Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. Immunity. **22**:247-257.

199. **Hakki, M. and A. P. Geballe**. 2005. Double-stranded RNA binding by human cytomegalovirus pTRS1. J. Virol. **79**:7311-7318.

200. **Haller, O., P. Staeheli, and G. Kochs**. 2007. Interferon-induced Mx proteins in antiviral host defense. Biochimie **89**:812-818.

201. **Haller, O., S. Stertz, and G. Kochs**. 2007. The Mx GTPase family of interferon-induced antiviral proteins. Microbes. Infect. **9**:1636-1643.

202. **Han, J. Q., G. Wroblewski, Z. Xu, R. H. Silverman, and D. J. Barton**. 2004. Sensitivity of hepatitis C virus RNA to the antiviral enzyme ribonuclease L is determined by a subset of efficient cleavage sites. J. Interferon Cytokine Res. **24**:664-676.

203. **Harber, J. and E. Wimmer**. 1993. Aspects of the molecular biology of picornaviruses In Regulation of Gene Expression in Animal Viruses., p. 189-224. *In* L. Carrasco, N. Sonenberg, and E. Wimmer (eds.), Proc. NATO ASI.

204. **Harte, M. T., I. R. Haga, G. Maloney, P. Gray, P. C. Reading, N. W. Bartlett, G. L. Smith, A. Bowie, and L. A. O'Neill**. 2003. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. J. Exp. Med. **197**:343-351.

205. **Harty, R. N., P. M. Pitha, and A. Okumura**. 2009. Antiviral Activity of Innate Immune Protein ISG15. J. Innate. Immun. **1**:397-404.

206. **Hassel, B. A., A. Zhou, C. Sotomayor, A. Maran, and R. H. Silverman**. 1993. A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon. EMBO J. **12**:3297-3304.

207. **Hayashi, Y., T. Ando, E. Utagawa, S. Sekine, S. Okada, K. Yabuuchi, T. Miki, and M. Ohashi**. 1989. Western blot (immunoblot) assay of small, round-structured virus associated with an acute gastroenteritis outbreak in Tokyo. J. Clin. Microbiol. **27**:1728-1733.

208. **Hayden, M. S. and S. Ghosh**. 2004. Signaling to NF-kappaB. Genes Dev. **18**:2195-2224.

209. **Hedger, R. S.** 1972. Foot-and-mouth disease and the African buffalo (Syncerus caffer). J. Comp Pathol. **82**:19-28.

210. **Heim, M. H.** 1999. The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. J. Recept. Signal. Transduct. Res. **19**:75-120.

211. **Helbig, K. J., D. T. Lau, L. Semendric, H. A. Harley, and M. R. Beard**. 2005. Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. Hepatology **42**:702-710.

212. **Hensley, S. E., A. K. Pinto, H. D. Hickman, R. J. Kastenmayer, J. R. Bennink, H. W. Virgin, and J. W. Yewdell**. 2009. Murine norovirus infection has no significant effect on adaptive immunity to vaccinia virus or influenza A virus. J. Virol. **83**:7357-7360.

213. **Herrmann, J. E., N. A. Nowak, and N. R. Blacklow**. 1985. Detection of Norwalk virus in stools by enzyme immunoassay. J. Med. Virol. **17**:127-133.

214. **Hilton, L., K. Moganeradj, G. Zhang, Y. H. Chen, R. E. Randall, J. W. McCauley, and S. Goodbourn**. 2006. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. J. Virol. **80**:11723-11732.

215. **Hinson, E. R. and P. Cresswell**. 2009. The N-terminal amphipathic alpha-helix of viperin mediates localization to the cytosolic face of the endoplasmic reticulum and inhibits protein secretion. J. Biol. Chem. **284**:4705-4712.

216. **Hinton, T. M., F. Li, and B. S. Crabb**. 2000. Internal ribosomal entry site-mediated translation initiation in equine rhinitis A virus: similarities to and differences from that of foot-and-mouth disease virus. J. Virol. **74**:11708-11716.

217. **Ho, L. J., L. F. Hung, C. Y. Weng, W. L. Wu, P. Chou, Y. L. Lin, D. M. Chang, T. Y. Tai, and J. H. Lai**. 2005. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J. Immunol. **174**:8163-8172.

218. **Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, K. Crozat, S. Sovath, J. Han, and B. Beutler**. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature **424**:743-748.

219. **Hofacker, I. L., M. Fekete, and P. F. Stadler**. 2002. Secondary structure prediction for aligned RNA sequences. J. Mol. Biol. **319**:1059-1066.

220. **Hofer, B., F. Steck, H. Gerber, J. Lohrer, J. Nicolet, and M. Paccaud**. 1973. An investigation of the etiology of viral respiratory disease in a remount depot, p. 103-109. *In* J. Bryans and H. Gerber (eds.), Equine infectious diseases.

221. **Holmes, D., M. Kemen, and L. Coggins**. 1978. Equine rhinovirus infection - serologic evidence of infection in selected United States horse populations *In* J. Bryans and H. Gerber (eds.), Equine infectious diseases IV.

222. **Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi**. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature **434**:772-777.

223. **Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann**. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science **314**:994-997.

224. **Horvath, C. M.** 2004. Weapons of STAT destruction. Interferon evasion by paramyxovirus V protein. Eur. J. Biochem. **271**:4621-4628.

225. **Houseley, J., J. LaCava, and D. Tollervey**. 2006. RNA-quality control by the exosome. Nat. Rev. Mol. Cell Biol. **7**:529-539.

226. **Hovanessian, A. G., R. E. Brown, and I. M. Kerr**. 1977. Synthesis of low molecular weight inhibitor of protein synthesis with enzyme from interferon-treated cells. Nature **268**:537-540.

227. **Hsu, C. C., C. E. Wobus, E. K. Steffen, L. K. Riley, and R. S. Livingston**. 2005. Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. Clin. Diagn. Lab Immunol. **12**:1145-1151.

228. **Hsu, C. C., L. K. Riley, H. M. Wills, and R. S. Livingston**. 2006. Persistent infection with and serologic cross-reactivity of three novel murine noroviruses. Comp Med. **56**:247-251.

229. **Hsu, C. C., L. K. Riley, and R. S. Livingston**. 2007. Molecular characterization of three novel murine noroviruses. Virus Genes **34**:147-155.

230. **Huang, Z., S. Krishnamurthy, A. Panda, and S. K. Samal**. 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J. Virol. **77**:8676-8685.

231. **Huthoff, H. and B. Berkhout**. 2002. Multiple secondary structure rearrangements during HIV-1 RNA dimerization. Biochemistry **41**:10439-10445.

232. **Hutson, A. M., R. L. Atmar, D. M. Marcus, and M. K. Estes**. 2003. Norwalk virus-like particle hemagglutination by binding to h histo-blood group antigens. J. Virol. **77**:405-415.

233. **Hutson, A. M., R. L. Atmar, and M. K. Estes**. 2004. Norovirus disease: changing epidemiology and host susceptibility factors. Trends Microbiol. **12**:279-287.

234. **Hyde, J. L., S. V. Sosnovtsev, K. Y. Green, C. Wobus, H. W. Virgin, and J. M. Mackenzie**. 2009. Mouse norovirus replication is associated with virus-induced vesicle clusters originating from membranes derived from the secretory pathway. J. Virol. **83** :9709-9719.

235. **Ihle, J. N.** 1995. The Janus protein tyrosine kinase family and its role in cytokine signaling. Adv. Immunol. **60**:1-35.

236. **Imani, F. and B. L. Jacobs**. 1988. Inhibitory activity for the interferon-induced protein kinase is associated with the reovirus serotype 1 sigma 3 protein. Proc. Natl. Acad. Sci. U. S. A **85**:7887-7891.

237. **Iordanov, M. S., J. D. Kirsch, O. P. Ryabinina, J. Wong, P. N. Spitz, V. B. Korcheva, A. Thorburn, and B. E. Magun**. 2005. Recruitment of TRADD, FADD, and caspase 8 to double-stranded RNA-triggered death inducing signaling complexes (dsRNA-DISCs). Apoptosis. **10**:167-176.

238. **Iqbal, M., E. Poole, S. Goodbourn, and J. W. McCauley**. 2004. Role for bovine viral diarrhea virus Erns glycoprotein in the control of activation of beta interferon by double-stranded RNA. J. Virol. **78**:136-145.

239. **Isaacs, A. and J. Lindenmann**. 1957. Virus interference. I. The interferon. Proc. R. Soc. Lond B Biol. Sci. **147**:258-267.

240. **Ito, T., M. Yang, and W. S. May**. 1999. RAX, a cellular activator for double-stranded RNA-dependent protein kinase during stress signaling. J. Biol. Chem. **274**:15427-15432.

241. **Jackson, W. T., T. H. Giddings, Jr., M. P. Taylor, S. Mulinyawe, M. Rabinovitch, R. R. Kopito, and K. Kirkegaard** . 2005. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS. Biol. **3**:e156.

242. **Jacobson, S. J., D. A. Konings, and P. Sarnow**. 1993. Biochemical and genetic evidence for a pseudoknot structure at the 3' terminus of the poliovirus RNA genome and its role in viral RNA amplification. J. Virol. **67**:2961-2971.

243. **Jang, S. K., T. V. Pestova, C. U. Hellen, G. W. Witherell, and E. Wimmer**. 1990. Cap-independent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. Enzyme **44**:292-309.

244. **Jang, S. K. and E. Wimmer**. 1990. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. **4**:1560-1572.

245. **Jaworska, J., A. Gravel, K. Fink, N. Grandvaux, and L. Flamand**. 2007. Inhibition of transcription of the beta interferon gene by the human herpesvirus 6 immediate-early 1 protein. J. Virol. **81**:5737-5748.

246. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes**. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. **66**:6527-6532.

247. **Jiang, X., J. Wang, D. Y. Graham, and M. K. Estes**. 1992. Detection of Norwalk virus in stool by polymerase chain reaction. J. Clin. Microbiol. **30**:2529-2534.

248. **Jiang, Z., T. W. Mak, G. Sen, and X. Li**. 2004. Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc. Natl. Acad. Sci. U. S. A **101**:3533-3538.

249. **Joachims, M., P. C. Van Breugel, and R. E. Lloyd**. 1999. Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. J. Virol. **73**:718-727.

250. **Joo, M., Y. S. Hahn, M. Kwon, R. T. Sadikot, T. S. Blackwell, and J. W. Christman**. 2005. Hepatitis C virus core protein suppresses NF-kappaB activation and cyclooxygenase-2 expression by direct interaction with IkappaB kinase beta. J. Virol. **79**:7648-7657.

251. **Jordan, W. S., Jr., I. Gordon, and W. R. Dorrance**. 1953. A study of illness in a group of Cleveland families. VII. Transmission of acute non-bacterial gastroenteritis to volunteers: evidence for two different etiologic agents. J. Exp. Med. **98**:461-475.

252. **Jounai, N., F. Takeshita, K. Kobiyama, A. Sawano, A. Miyawaki, K. Q. Xin, K. J. Ishii, T. Kawai, S. Akira, K. Suzuki, and K. Okuda**. 2007. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. Proc. Natl. Acad. Sci. U. S. A **104** :14050-14055.

253. **Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu**. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. **194**:863-869.

254. **Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama**. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. **41**:1548-1557.

255. **Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R. M. Chanock**. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J. Virol. **10**:1075-1081.

256. **Kapikian, A. Z.** 2000. The discovery of the 27-nm Norwalk virus: an historic perspective. J. Infect. Dis. **181 Suppl 2**:S295-S302.

257. **Kaplan, J. E., R. Feldman, D. S. Campbell, C. Lookabaugh, and G. W. Gary**. 1982. The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. Am. J. Public Health **72**:1329-1332.

258. **Kaplan, J. E., G. W. Gary, R. C. Baron, N. Singh, L. B. Schonberger, R. Feldman, and H. B. Greenberg**. 1982. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann. Intern. Med. **96**:756-761.

259. **Karst, S. M., C. E. Wobus, M. Lay, J. Davidson, and H. W. Virgin**. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science **299**:1575-1578.

260. **Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira**. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature **441**:101-105.

261. **Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira**. 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J. Exp. Med. **205**:1601-1610.

262. **Katow, S. and A. Sugiura**. 1988. Low pH-induced conformational change of rubella virus envelope proteins. J. Gen. Virol. **69 (Pt 11)**:2797-2807.

263. **Katpally, U., C. E. Wobus, K. Dryden, H. W. Virgin, and T. J. Smith**. 2008. Structure of antibody-neutralized murine norovirus and unexpected differences from viruslike particles. J. Virol. **82**:2079-2088.

264. **Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira**. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. **6**:981-988.

265. **Kawakami, T., M. Matsumoto, M. Sato, H. Harada, T. Taniguchi, and M. Kitagawa**. 1995. Possible involvement of the transcription factor ISGF3 gamma in virus-induced expression of the IFN-beta gene. FEBS Lett. **358**:225-229.

266. **Kawakubo, K. and C. E. Samuel**. 2000. Human RNA-specific adenosine deaminase (ADAR1) gene specifies transcripts that initiate from a constitutively active alternative promoter. Gene **258**:165-172.

267. **Kerr, I. M. and R. E. Brown**. 1978. pppA2'p5'A2'p5'A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc. Natl. Acad. Sci. U. S. A **75**:256-260.

268. **Kerscher, O., R. Felberbaum, and M. Hochstrasser**. 2006. Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu. Rev. Cell Dev. Biol. **22**:159-180.

269. **Khabar, K. S., F. Al-Zoghaibi, M. N. Al-Ahdal, T. Murayama, M. Dhalla, N. Mukaida, M. Taha, S. T. Al-Sedairy, Y. Siddiqui, G. Kessie, and K. Matsushima**. 1997. The alpha chemokine, interleukin 8, inhibits the antiviral action of interferon alpha. J. Exp. Med. **186**:1077-1085.

270. **Kieft, J. S., K. Zhou, R. Jubin, M. G. Murray, J. Y. Lau, and J. A. Doudna**. 1999. The hepatitis C virus internal ribosome entry site adopts an ion-dependent tertiary fold. J. Mol. Biol. **292**:513-529.

271. **Kim, T. S. and S. Perlman**. 2003. Protection against CTL escape and clinical disease in a murine model of virus persistence. J. Immunol. **171**:2006-2013.

272. **Kint, T., R. Goldsby, and B. Osbourne**. 2007. Innate immunity, p. 52-75. Kuby Immunology.

273. **Kirchweger, R., E. Ziegler, B. J. Lamphear, D. Waters, H. D. Liebig, W. Sommergruber, F. Sobrino, C. Hohenadl, D. Blaas, R. E. Rhoads, and .** 1994. Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4 gamma. J. Virol. **68**:5677-5684.

274. **Kirkegaard, K., M. P. Taylor, and W. T. Jackson**. 2004. Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat. Rev. Microbiol. **2**:301-314.

275. **Knudsen, B. and J. Hein**. 1999. RNA secondary structure prediction using stochastic context-free grammars and evolutionary history. Bioinformatics. **15**:446-454.

276. **Ko, G., Z. D. Jiang, P. C. Okhuysen, and H. L. DuPont**. 2006. Fecal cytokines and markers of intestinal inflammation in international travelers with diarrhea due to Noroviruses. J. Med. Virol. **78**:825-828.

277. **Kochs, G. and O. Haller**. 1999. Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. Proc. Natl. Acad. Sci. U. S. A **96**:2082-2086.

278. **Kolupaeva, V. G., I. B. Lomakin, T. V. Pestova, and C. U. Hellen**. 2003. Eukaryotic initiation factors 4G and 4A mediate conformational changes downstream of the initiation codon of the encephalomyocarditis virus internal ribosomal entry site. Mol. Cell Biol. **23**:687-698.

279. **Kolykhalov, A. A., S. M. Feinstone, and C. M. Rice**. 1996. Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. J. Virol. **70**:3363-3371.

280. **Kolykhalov, A. A., K. Mihalik, S. M. Feinstone, and C. M. Rice**. 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo. J. Virol. **74**:2046-2051.

281. **Kopecky-Bromberg, S. A., L. Martinez-Sobrido, M. Frieman, R. A. Baric, and P. Palese**. 2007. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. J. Virol. **81** :548-557.

282. **Kotenko, S. V., L. S. Izotova, O. V. Mirochnitchenko, E. Esterova, H. Dickensheets, R. P. Donnelly, and S. Pestka**. 2001. Identification, cloning, and characterization of a novel soluble receptor that binds IL-22 and neutralizes its activity. J. Immunol. **166**:7096-7103.

283. **Krausslich, H. G., C. Holscher, Q. Reuer, J. Harber, and E. Wimmer**. 1990. Myristoylation of the poliovirus polyprotein is required for proteolytic processing of the capsid and for viral infectivity. J. Virol. **64**:2433-2436.

284. **Kriegshauser, G., A. Deutz, E. Kuechler, T. Skern, H. Lussy, and N. Nowotny**. 2005. Prevalence of neutralizing antibodies to Equine rhinitis A and B virus in horses and man. Vet. Microbiol. **106**:293-296.

285. **Kubota, K., K. Nakahara, T. Ohtsuka, S. Yoshida, J. Kawaguchi, Y. Fujita, Y. Ozeki, A. Hara, C. Yoshimura, H. Furukawa, H. Haruyama, K. Ichikawa, M. Yamashita, T. Matsuoka, and Y. Iijima**. 2004. Identification of 2'-phosphodiesterase, which plays a role in the 2-5A system regulated by interferon. J. Biol. Chem. **279**:37832-37841.

286. **Kubota, T., N. Yokosawa, S. Yokota, and N. Fujii**. 2001. C terminal CYS-RICH region of mumps virus structural V protein correlates with block of interferon alpha and gamma signal transduction pathway through decrease of STAT 1-alpha. Biochem. Biophys. Res. Commun. **283**:255-259.

287. **Kubota, T., N. Yokosawa, S. Yokota, and N. Fujii**. 2002. Association of mumps virus V protein with RACK1 results in dissociation of STAT-1 from the alpha interferon receptor complex. J. Virol. **76**:12676-12682.

288. **Kubota, T., N. Yokosawa, S. Yokota, N. Fujii, M. Tashiro, and A. Kato**. 2005. Mumps virus V protein antagonizes interferon without the complete degradation of STAT1. J. Virol. **79**:4451-4459.

289. **Kuhn, R.** 2007. Togaviridae: the viruses and their replication, p. 1001-1022. *In* D. Knipe and P. Howley (eds.), Field's Virology.

290. **Kujala, P., A. Ikaheimonen, N. Ehsani, H. Vihinen, P. Auvinen, and L. Kaariainen**. 2001. Biogenesis of the Semliki Forest virus RNA replication complex. J. Virol. **75**:3873-3884.

291. **Kumar, H., T. Kawai, H. Kato, S. Sato, K. Takahashi, C. Coban, M. Yamamoto, S. Uematsu, K. J. Ishii, O. Takeuchi, and S. Akira**. 2006.

Essential role of IPS-1 in innate immune responses against RNA viruses. J. Exp. Med. **203**:1795-1803.

292. **L'Homme, Y., R. Sansregret, E. Plante-Fortier, A. M. Lamontagne, G. Lacroix, M. Ouardani, J. Deschamps, G. Simard, and C. Simard**. 2009. Genetic diversity of porcine Norovirus and Sapovirus: Canada, 2005-2007. Arch. Virol. **154**:581-593.

293. **Langland, J. O., S. Pettiford, B. Jiang, and B. L. Jacobs**. 1994. Products of the porcine group C rotavirus NSP3 gene bind specifically to double-stranded RNA and inhibit activation of the interferon-induced protein kinase PKR. J. Virol. **68**:3821-3829.

294. **Langland, J. O., J. M. Cameron, M. C. Heck, J. K. Jancovich, and B. L. Jacobs**. 2006. Inhibition of PKR by RNA and DNA viruses. Virus Res. **119**:100-110.

295. **Le Bon. A. and D. F. Tough**. 2002. Links between innate and adaptive immunity via type I interferon. Curr. Opin. Immunol. **14**:432-436.

296. **Le Bon. A., N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough**. 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat. Immunol. **4**:1009-1015.

297. **Le Guyader. F., M. K. Estes, M. E. Hardy, F. H. Neill, J. Green, D. W. Brown, and R. L. Atmar**. 1996. Evaluation of a degenerate primer for the PCR detection of human caliciviruses. Arch. Virol. **141**:2225-2235.

298. **Lee, H. K., J. M. Lund, B. Ramanathan, N. Mizushima, and A. Iwasaki**. 2007. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science **315**:1398-1401.

299. **Lee, S. B. and M. Esteban**. 1994. The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology **199**:491-496.

300. **Lee, T. G., J. Tomita, A. G. Hovanessian, and M. G. Katze**. 1992. Characterization and regulation of the 58,000-dalton cellular inhibitor of the interferon-induced, dsRNA-activated protein kinase. J. Biol. Chem. **267**:14238-14243.

301. **Lee, Y. F., A. Nomoto, B. M. Detjen, and E. Wimmer**. 1977. A protein covalently linked to poliovirus genome RNA. Proc. Natl. Acad. Sci. U. S. A **74**:59-63.

302. **Lenschow, D. J., C. Lai, N. Frias-Staheli, N. V. Giannakopoulos, A. Lutz, T. Wolff, A. Osiak, B. Levine, R. E. Schmidt, A. Garcia-Sastre, D. A. Leib, A. Pekosz, K. P. Knobeloch, I. Horak, and H. W. Virgin**. 2007. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. Proc. Natl. Acad. Sci. U. S. A **104**:1371-1376.

303. **Leonard, G. T. and G. C. Sen**. 1996. Effects of adenovirus E1A protein on interferon-signaling. Virology **224**:25-33.

304. **Li, C., C. Z. Ni, M. L. Havert, E. Cabezas, J. He, D. Kaiser, J. C. Reed, A. C. Satterthwait, G. Cheng, and K. R. Ely**. 2002. Downstream regulator TANK binds to the CD40 recognition site on TRAF3. Structure. **10**:403-411.

305. **Li, F., G. F. Browning, M. J. Studdert, and B. S. Crabb**. 1996. Equine rhinovirus 1 is more closely related to foot-and-mouth disease virus than to other picornaviruses. Proc. Natl. Acad. Sci. U. S. A **93**:990-995.

306. **Li, K., E. Foy, J. C. Ferreon, M. Nakamura, A. C. Ferreon, M. Ikeda, S. C. Ray, M. Gale, Jr., and S. M. Lemon**. 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc. Natl. Acad. Sci. U. S. A **102**:2992-2997.

307. **Li, Q., R. Means, S. Lang, and J. U. Jung**. 2007. Downregulation of gamma interferon receptor 1 by Kaposi's sarcoma-associated herpesvirus K3 and K5. J. Virol. **81**:2117-2127.

308. **Li, S., S. Labrecque, M. C. Gauzzi, A. R. Cuddihy, A. H. Wong, S. Pellegrini, G. J. Matlashewski, and A. E. Koromilas**. 1999. The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. Oncogene **18**:5727-5737.

309. **Li, S., J. Y. Min, R. M. Krug, and G. C. Sen**. 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology **349**:13-21.

310. **Li, X., C. T. Ranjith-Kumar, M. T. Brooks, S. Dharmaiah, A. B. Herr, C. Kao, and P. Li**. 2009. The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA. J. Biol. Chem. **284**:13881-13891.

311. **Li, X., C. Lu, M. Stewart, H. Xu, R. K. Strong, T. Igumenova, and P. Li**. 2009. Structural Basis of Double-stranded RNA Recognition by the RIG-I like Receptor MDA5. Arch. Biochem. Biophys.

312. **Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott**. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol. Cell Biol. **18**:2986-2996.

313. **Lin, R., P. Genin, Y. Mamane, M. Sgarbanti, A. Battistini, W. J. Harrington, Jr., G. N. Barber, and J. Hiscott**. 2001. HHV-8 encoded vIRF-1 represses the interferon antiviral response by blocking IRF-3 recruitment of the CBP/p300 coactivators. Oncogene **20**:800-811.

314. **Lin, R., R. S. Noyce, S. E. Collins, R. D. Everett, and K. L. Mossman**. 2004. The herpes simplex virus ICP0 RING finger domain inhibits. J. Virol. **78**:1675-1684.

315. **Lin, R., J. Lacoste, P. Nakhaei, Q. Sun, L. Yang, S. Paz, P. Wilkinson, I. Julkunen, D. Vitour, E. Meurs, and J. Hiscott**. 2006. Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. J. Virol. **80**:6072-6083.

316. **Lin, R. J., C. L. Liao, E. Lin, and Y. L. Lin**. 2004. Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. J. Virol. **78**:9285-9294.

317. **Lin, W., S. S. Kim, E. Yeung, Y. Kamegaya, J. T. Blackard, K. A. Kim, M. J. Holtzman, and R. T. Chung**. 2006. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. J. Virol. **80**:9226-9235.

318. **Lindenbach, T. R.** 2009. Flaviviridae, p. 1101-1152. *In* D. Knipe and P. Howley (eds.), Fields Virology.

319. **Lindenmann, J.** 1962. Resistance of mice to mouse-adapted influenza A virus. Virology **16**:203-204.

320. **Lindesmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. LePendu, and R. Baric**. 2003. Human susceptibility and resistance to Norwalk virus infection. Nat. Med. **9**:548-553.

321. **Lindesmith, L., C. Moe, J. LePendu, J. A. Frelinger, J. Treanor, and R. S. Baric**. 2005. Cellular and humoral immunity following Snow Mountain virus challenge. J. Virol. **79**:2900-2909.

322. **Liu, B., I. N. Clarke, and P. R. Lambden**. 1996. Polyprotein processing in Southampton virus: identification of 3C-like protease cleavage sites by in vitro mutagenesis. J. Virol. **70**:2605-2610.

323. **Liu, B., A. M. Woltman, H. L. Janssen, and A. Boonstra**. 2009. Modulation of dendritic cell function by persistent viruses. J. Leukoc. Biol. **85**:205-214.

324. **Liu, B. L., P. R. Lambden, H. Gunther, P. Otto, M. Elschner, and I. N. Clarke**. 1999. Molecular characterization of a bovine enteric calicivirus: relationship to the Norwalk-like viruses. J. Virol. **73**:819-825.

325. **Liu, G., S. M. Kahan, Y. Jia, and S. M. Karst**. 2009. Primary high-dose murine norovirus 1 infection fails to protect from secondary challenge with homologous virus. J. Virol. **83**:6963-6968.

326. **Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor, and G. J. Hannon**. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science **305**:1437-1441.

327. **Liu, Y. C., J. Penninger, and M. Karin**. 2005. Immunity by ubiquitylation: a reversible process of modification. Nat. Rev. Immunol. **5**:941-952.

328. **Liu, Z., M. Winkler, and B. Biegalke**. 2009. Human cytomegalovirus: host immune modulation by the viral US3 gene. Int. J. Biochem. Cell Biol. **41**:503-506.

329. **LoBue, A. D., J. M. Thompson, L. Lindesmith, R. E. Johnston, and R. S. Baric**. 2009. Alphavirus-adjuvanted norovirus-like particle vaccines: heterologous, humoral, and mucosal immune responses protect against murine norovirus challenge. J. Virol. **83** :3212-3227.

330. **Lochridge, V. P., K. L. Jutila, J. W. Graff, and M. E. Hardy**. 2005. Epitopes in the P2 domain of norovirus VP1 recognized by monoclonal antibodies that block cell interactions. J. Gen. Virol. **86**:2799-2806.

331. **Lochridge, V. P. and M. E. Hardy** . 2007. A single-amino-acid substitution in the P2 domain of VP1 of murine norovirus is sufficient for escape from antibody neutralization. J. Virol. **81**:12316-12322.

332. **Loeb, K. R. and A. L. Haas**. 1992. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. J. Biol. Chem. **267**:7806-7813.

333. **Look, D. C., W. T. Roswit, A. G. Frick, Y. Gris-Alevy, D. M. Dickhaus, M. J. Walter, and M. J. Holtzman**. 1998. Direct suppression of Stat1 function during adenoviral infection. Immunity. **9**:871-880.

334. **Lopman, B. A., M. H. Reacher, I. B. Vipond, J. Sarangi, and D. W. Brown**. 2004. Clinical manifestation of norovirus gastroenteritis in health care settings. Clin. Infect. Dis. **39**:318-324.

335. **Lu, G., J. T. Reinert, I. Pitha-Rowe, A. Okumura, M. Kellum, K. P. Knobeloch, B. Hassel, and P. M. Pitha**. 2006. ISG15 enhances the innate

antiviral response by inhibition of IRF-3 degradation. Cell Mol. Biol. (Noisy. -le-grand) **52**:29-41.

336. **Lukavsky, P. J.** 2009. Structure and function of HCV IRES domains. Virus Res. **139**:166-171.

337. **Malakhov, M. P., O. A. Malakhova, K. I. Kim, K. J. Ritchie, and D. E. Zhang**. 2002. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J. Biol. Chem. **277** :9976-9981.

338. **Malathi, K., J. M. Paranjape, E. Bulanova, M. Shim, J. M. Guenther-Johnson, P. W. Faber, T. E. Eling, B. R. Williams, and R. H. Silverman**. 2005. A transcriptional signaling pathway in the IFN system mediated by 2'-5'-oligoadenylate activation of RNase L. Proc. Natl. Acad. Sci. U. S. A **102**:14533-14538.

339. **Manche, L., S. R. Green, C. Schmedt, and M. B. Mathews**. 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell Biol. **12**:5238-5248.

340. **Maniatis, T., J. V. Falvo, T. H. Kim, T. K. Kim, C. H. Lin, B. S. Parekh, and M. G. Wathelet**. 1998. Structure and function of the interferon-beta enhanceosome. Cold Spring Harb. Symp. Quant. Biol. **63**:609-620.

341. **Marcus, P. I. and M. J. Sekellick**. 1977. Defective interfering particles with covalently linked [+/-]RNA induce interferon. Nature **266**:815-819.

342. **Marcus, P. I.** 1983. Interferon induction by viruses: one molecule of dsRNA as the threshold for interferon induction. Interferon **5**:115-180.

343. **Marie, I., J. E. Durbin, and D. E. Levy**. 1998. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. EMBO J. **17**:6660-6669.

344. **Markham, N. R. and M. Zuker**. 2008. UNAFold: software for nucleic acid folding and hybridization. Methods Mol. Biol. **453**:3-31.

345. **Marsh, M., A. Helenius, K. Matlin, and K. Simons**. 1983. Binding, endocytosis, and degradation of enveloped animal viruses. Methods Enzymol. **98**:260-266.

346. **Martinand, C., T. Salehzada, M. Silhol, B. Lebleu, and C. Bisbal**. 1998. RNase L inhibitor (RLI) antisense constructions block partially the down regulation of the 2-5A/RNase L pathway in encephalomyocarditis-virus-(EMCV)-infected cells. Eur. J. Biochem. **254**:248-255.

347. **Martinez-Costas, J., C. Gonzalez-Lopez, V. N. Vakharia, and J. Benavente**. 2000. Possible involvement of the double-stranded RNA-binding core protein sigmaA in the resistance of avian reovirus to interferon. J. Virol. **74**:1124-1131.

348. **Martinez-Salas, E., J. C. Saiz, M. Davila, G. J. Belsham, and E. Domingo**. 1993. A single nucleotide substitution in the internal ribosome entry site of foot-and-mouth disease virus leads to enhanced cap-independent translation in vivo. J. Virol. **67** :3748-3755.

349. **Mason, P. W., S. V. Bezborodova, and T. M. Henry**. 2002. Identification and characterization of a cis-acting replication element (cre) adjacent to the internal ribosome entry site of foot-and-mouth disease virus. J. Virol. **76**:9686-9694.

350. **Mathews, D. H.** 2004. Using an RNA secondary structure partition function to determine confidence in base pairs predicted by free energy minimization. RNA. **10**:1178-1190.

351. **Mathews, D. H., M. D. Disney, J. L. Childs, S. J. Schroeder, M. Zuker, and D. H. Turner**. 2004. Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc. Natl. Acad. Sci. U. S. A **101**:7287-7292.

352. **Mathews, D. H. and D. H. Turner**. 2006. Prediction of RNA secondary structure by free energy minimization. Curr. Opin. Struct. Biol. **16**:270-278.

353. **Matsui, S. M., J. P. Kim, H. B. Greenberg, W. Su, Q. Sun, P. C. Johnson, H. L. DuPont, L. S. Oshiro, and G. R. Reyes**. 1991. The isolation and characterization of a Norwalk virus-specific cDNA. J. Clin. Invest **87**:1456-1461.

354. **Matsumoto, M., S. Kikkawa, M. Kohase, K. Miyake, and T. Seya**. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem. Biophys. Res. Commun. **293**:1364-1369.

355. **Matzener, P., I. Magkouras, T. Rumenapf, E. Peterhans, and M. Schweizer**. 2009. The viral RNase E(rns) prevents IFN type-I triggering by pestiviral single- and double-stranded RNAs. Virus Res. **140**:15-23.

356. **Mauroy, A., A. Scipioni, E. Mathijs, C. Saegerman, J. Mast, J. C. Bridger, D. Ziant, C. Thys, and E. Thiry**. 2009. Epidemiological study of bovine norovirus infection by RT-PCR and a VLP-based antibody ELISA. Vet. Microbiol. **137**:243-251.

357. **McCartney, S. A., L. B. Thackray, L. Gitlin, S. Gilfillan, H. W. Virgin, and M. Colonna**. 2008. MDA-5 recognition of a murine norovirus. PLoS. Pathog. **4**:e1000108.

358. **McCaskill, J. S.** 1990. The equilibrium partition function and base pair binding probabilities for RNA secondary structure. Biopolymers **29**:1105-1119.

359. McCollum, W. H and Timoney, P. Studies on the seroprevalence and the frequency of equine rhinovirus-I and II infection in normal horse urine. Proc.6th Equine.Infect.Dis.Conf. 83-88. 1992.
Ref Type: Generic

360. **McKenna, K., A. S. Beignon, and N. Bhardwaj**. 2005. Plasmacytoid dendritic cells: linking innate and adaptive immunity. J. Virol. **79**:17-27.

361. **Melen, K., T. Ronni, B. Broni, R. M. Krug, C. H. von Bonsdorff, and I. Julkunen**. 1992. Interferon-induced Mx proteins form oligomers and contain a putative leucine zipper. J. Biol. Chem. **267**:25898-25907.

362. **Merika, M. and D. Thanos**. 2001. Enhanceosomes. Curr. Opin. Genet. Dev. **11**:205-208.

363. **Meylan, E., K. Burns, K. Hofmann, V. Blancheteau, F. Martinon, M. Kelliher, and J. Tschopp**. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat. Immunol. **5**:503-507.

364. **Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp**. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437**:1167-1172.

365. **Mibayashi, M., L. Martinez-Sobrido, Y. M. Loo, W. B. Cardenas, M. Gale, Jr., and A. Garcia-Sastre**. 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. **81**:514-524.

366. **Miller, D. M., B. M. Rahill, J. M. Boss, M. D. Lairmore, J. E. Durbin, J. W. Waldman, and D. D. Sedmak**. 1998. Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. J. Exp. Med. **187**:675-683.

367. **Min, J. Y. and R. M. Krug**. 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. Proc. Natl. Acad. Sci. U. S. A **103**:7100-7105.

368. **Minks, M. A., D. K. West, S. Benvin, and C. Baglioni**. 1979. Structural requirements of double-stranded RNA for the activation of 2',5'-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J. Biol. Chem. **254**:10180-10183.

369. **Moe, C. L., J. Gentsch, T. Ando, G. Grohmann, S. S. Monroe, X. Jiang, J. Wang, M. K. Estes, Y. Seto, C. Humphrey, and .** 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J. Clin. Microbiol. **32**:642-648.

370. **Moradpour, D., F. Penin, and C. M. Rice**. 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol. **5**:453-463.

371. **Mounts, A. W., T. Ando, M. Koopmans, J. S. Bresee, J. Noel, and R. I. Glass**. 2000. Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. J. Infect. Dis. **181 Suppl 2**:S284-S287.

372. **Mueller, N. H., D. H. Gilden, R. J. Cohrs, R. Mahalingam, and M. A. Nagel**. 2008. Varicella zoster virus infection: clinical features, molecular pathogenesis of disease, and latency. Neurol. Clin. **26**:675-97, viii.

373. **Muller, B., U. Klemm, M. A. Mas, and E. Schreier**. 2007. Genetic diversity and recombination of murine noroviruses in immunocompromised mice. Arch. Virol. **152**:1709-1719.

374. **Mumphrey, S. M., H. Changotra, T. N. Moore, E. R. Heimann-Nichols, C. E. Wobus, M. J. Reilly, M. Moghadamfalahi, D. Shukla, and S. M. Karst**. 2007. Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. J. Virol. **81**:3251-3263.

375. **Munoz-Fontela, C., M. A. Garcia, I. Garcia-Cao, M. Collado, J. Arroyo, M. Esteban, M. Serrano, and C. Rivas**. 2005. Resistance to viral infection of super p53 mice. Oncogene **24**:3059-3062.

376. **Munoz-Jordan, J. L., G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre**. 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. U. S. A **100**:14333-14338.

377. **Najarro, P., P. Traktman, and J. A. Lewis**. 2001. Vaccinia virus blocks gamma interferon signal transduction: viral VH1 phosphatase reverses Stat1 activation. J. Virol. **75**:3185-3196.

378. **Nakamura, M., H. Saito, and T. Hibi**. 2008. Advances in genomic research on hepatitis C virus with a useful tool, replicon system. Keio J. Med. **57**:75-83.

379. **Nanda, S. K. and M. D. Baron**. 2006. Rinderpest virus blocks type I and type II interferon action: role of structural and nonstructural proteins. J. Virol. **80**:7555-7568.

380. **Napoli, C., C. Lemieux, and R. Jorgensen**. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell **2**:279-289.

381. **Neill, J. D. and W. L. Mengeling** . 1988. Further characterization of the virus-specific RNAs in feline calicivirus infected cells. Virus Res. **11**:59-72.

382. **Nguyen, K. B., T. P. Salazar-Mather, M. Y. Dalod, J. B. Van Deusen, X. Q. Wei, F. Y. Liew, M. A. Caligiuri, J. E. Durbin, and C. A. Biron**. 2002. Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. J. Immunol. **169**:4279-4287.

383. **Nilsson, M., K. O. Hedlund, M. Thorhagen, G. Larson, K. Johansen, A. Ekspong, and L. Svensson**. 2003. Evolution of human calicivirus RNA in vivo: accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. J. Virol. **77**:13117-13124.

384. **Nomoto, A., N. Kitamura, F. Golini, and E. Wimmer**. 1977. The 5'-terminal structures of poliovirion RNA and poliovirus mRNA differ only in the genome-linked protein VPg. Proc. Natl. Acad. Sci. U. S. A **74**:5345-5349.

385. **Novak, J. E. and K. Kirkegaard**. 1991. Improved method for detecting poliovirus negative strands used to demonstrate specificity of positive-strand encapsidation and the ratio of positive to negative strands in infected cells. J. Virol. **65**:3384-3387.

386. **Novick, D., B. Cohen, and M. Rubinstein**. 1994. The human interferon alpha/beta receptor: characterization and molecular cloning. Cell **77**:391-400.

387. **Nussinov, R. and A. B. Jacobson**. 1980. Fast algorithm for predicting the secondary structure of single-stranded RNA. Proc. Natl. Acad. Sci. U. S. A **77**:6309-6313.

388. **O'Donnell, V., M. Larocco, and B. Baxt**. 2008. Heparan sulfate-binding foot-and-mouth disease virus enters cells via caveola-mediated endocytosis. J. Virol. **82**:9075-9085.

389. **Offermann, M. K.** 2007. Kaposi sarcoma herpesvirus-encoded interferon regulator factors. Curr. Top. Microbiol. Immunol. **312**:185-209.

390. **Ohga, S., A. Nomura, H. Takada, and T. Hara**. 2002. Immunological aspects of Epstein-Barr virus infection. Crit Rev. Oncol. Hematol. **44**:203-215.

391. **Oldstone, M. B. and F. J. Dixon**. 1968. Direct immunofluorescent tissue culture assay for lymphocytic choriomeningitis virus. J. Immunol. **100**:1135-1138.

392. **Oldstone, M. B.** 2006. Viral persistence: parameters, mechanisms and future predictions. Virology **344**:111-118.

393. **Oldstone, M. B.** 2009. Anatomy of viral persistence. PLoS. Pathog. **5**:e1000523.

394. **Oliver, S. L., A. M. Dastjerdi, S. Wong, L. El-Attar, C. Gallimore, D. W. Brown, J. Green, and J. C. Bridger**. 2003. Molecular characterization of

bovine enteric caliciviruses: a distinct third genogroup of noroviruses (Norwalk-like viruses) unlikely to be of risk to humans. J. Virol. **77**:2789-2798.

395. **Oliver, S. L., E. Asobayire, A. M. Dastjerdi, and J. C. Bridger**. 2006. Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae. Virology **350**:240-250.

396. **Osiak, A., O. Utermohlen, S. Niendorf, I. Horak, and K. P. Knobeloch**. 2005. ISG15, an interferon-stimulated ubiquitin-like protein, is not essential for STAT1 signaling and responses against vesicular stomatitis and lymphocytic choriomeningitis virus. Mol. Cell Biol. **25**:6338-6345.

397. **Ou, J. H., E. G. Strauss, and J. H. Strauss**. 1981. Comparative studies of the 3'-terminal sequences of several alpha virus RNAs. Virology **109**:281-289.

398. **Ou, J. H., C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss**. 1982. Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. Proc. Natl. Acad. Sci. U. S. A **79**:5235-5239.

399. **Ou, J. H., E. G. Strauss, and J. H. Strauss**. 1983. The 5'-terminal sequences of the genomic RNAs of several alphaviruses. J. Mol. Biol. **168**:1-15.

400. **Palmenberg, A. C., G. D. Parks, D. J. Hall, R. H. Ingraham, T. W. Seng, and P. V. Pallai**. 1992. Proteolytic processing of the cardioviral P2 region: primary 2A/2B cleavage in clone-derived precursors. Virology **190**:754-762.

401. **Palosaari, H., J. P. Parisien, J. J. Rodriguez, C. M. Ulane, and C. M. Horvath**. 2003. STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. J. Virol. **77**:7635-7644.

402. **Pampin, M., Y. Simonin, B. Blondel, Y. Percherancier, and M. K. Chelbi-Alix**. 2006. Cross talk between PML and p53 during poliovirus infection: implications for antiviral defense. J. Virol. **80**:8582-8592.

403. **Pang, X., B. Lee, L. Chui, J. K. Preiksaitis, and S. S. Monroe**. 2004. Evaluation and validation of real-time reverse transcription-pcr assay using the LightCycler system for detection and quantitation of norovirus. J. Clin. Microbiol. **42**:4679-4685.

404. **Panne, D., T. Maniatis, and S. C. Harrison**. 2004. Crystal structure of ATF-2/c-Jun and IRF-3 bound to the interferon-beta enhancer. EMBO J. **23**:4384-4393.

405. **Panne, D., S. M. McWhirter, T. Maniatis, and S. C. Harrison**. 2007. Interferon regulatory factor 3 is regulated by a dual phosphorylation-dependent switch. J. Biol. Chem. **282**:22816-22822.

406. **Panne, D., T. Maniatis, and S. C. Harrison**. 2007. An atomic model of the interferon-beta enhanceosome. Cell **129**:1111-1123.

407. **Parrino, T. A., D. S. Schreiber, J. S. Trier, A. Z. Kapikian, and N. R. Blacklow**. 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. N. Engl. J. Med. **297**:86-89.

408. **Patel, M. M., A. J. Hall, J. Vinje, and U. D. Parashar**. 2009. Noroviruses: a comprehensive review. J. Clin. Virol. **44**:1-8.

409. **Patel, R. C. and G. C. Sen**. 1998. PACT, a protein activator of the interferon-induced protein kinase, PKR. EMBO J. **17**:4379-4390.

410. **Pathak, H. B., J. J. Arnold, P. N. Wiegand, M. R. Hargittai, and C. E. Cameron**. 2007. Picornavirus genome replication: assembly and organization of the VPg uridylylation ribonucleoprotein (initiation) complex. J. Biol. Chem. **282**:16202-16213.

411. **Patterson, J. B. and C. E. Samuel**. 1995. Expression and regulation by interferon of a double-stranded-RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. Mol. Cell Biol. **15**:5376-5388.

412. **Paul, A. V., E. Rieder, D. W. Kim, J. H. van Boom, and E. Wimmer**. 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. J. Virol. **74**:10359-10370.

413. **Paulus, C., S. Krauss, and M. Nevels**. 2006. A human cytomegalovirus antagonist of type I IFN-dependent signal transducer and activator of transcription signaling. Proc. Natl. Acad. Sci. U. S. A **103**:3840-3845.

414. **Paun, A. and P. M. Pitha**. 2007. The IRF family, revisited. Biochimie **89**:744-753.

415. **Pedersen, K. W., M. Y. van der, N. Roos, and E. J. Snijder**. 1999. Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. J. Virol. **73**:2016-2026.

416. **Pelletier, J. and N. Sonenberg**. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature **334**:320-325.

417. **Perdue, K. A., K. Y. Green, M. Copeland, E. Barron, M. Mandel, L. J. Faucette, E. M. Williams, S. V. Sosnovtsev, W. R. Elkins, and J. M. Ward**. 2007. Naturally occurring murine norovirus infection in a large research institution. J. Am. Assoc. Lab Anim Sci. **46**:39-45.

418. **Perry, J. W., S. Taube, and C. E. Wobus**. 2009. Murine norovirus-1 entry into permissive macrophages and dendritic cells is pH-independent. Virus Res. **143**:125-129.

419. **Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and Reis e Sousa**. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science **314**:997-1001.

420. **Planz, O., S. Pleschka, and T. Wolff**. 2009. Borna disease virus: a unique pathogen and its interaction with intracellular signalling pathways. Cell Microbiol. **11**:872-879.

421. **Platanias, L. C.** 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat. Rev. Immunol. **5**:375-386.

422. **Plummer, G. and J. Kerry**. 1962. Studies on an equine respiratory virus. Vet. Rec **74**:967-970.

423. **Plummer, G.** 1962. An equine respiratory virus with enterovirus properties. Nature **195**:519-520.

424. **Plummer, G.** 1963. An equine respiratory enterovirus. Some biological and physical properties. Arch. Gesamte Virusforsch. **12**:694-700.

425. **Pomerantz, J. L. and D. Baltimore**. 1999. NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. EMBO J. **18**:6694-6704.

426. **Poppers, J., M. Mulvey, C. Perez, D. Khoo, and I. Mohr**. 2003. Identification of a lytic-cycle Epstein-Barr virus gene product that can regulate PKR activation. J. Virol. **77**:228-236.

427. **Porter, F. W., Y. A. Bochkov, A. J. Albee, C. Wiese, and A. C. Palmenberg**. 2006. A picornavirus protein interacts with Ran-GTPase and disrupts nucleocytoplasmic transport. Proc. Natl. Acad. Sci. U. S. A **103**:12417-12422.

428. **Powell, P. P., L. K. Dixon, and R. M. Parkhouse**. 1996. An IkappaB homolog encoded by African swine fever virus provides a novel mechanism for downregulation of proinflammatory cytokine responses in host macrophages. J. Virol. **70**:8527-8533.

429. **Prasad, B. V., R. Rothnagel, X. Jiang, and M. K. Estes**. 1994. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. J. Virol. **68**:5117-5125.

430. **Prasad, B. V., M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann, and M. K. Estes**. 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science **286**:287-290.

431. **Prentice, E., W. G. Jerome, T. Yoshimori, N. Mizushima, and M. R. Denison**. 2004. Coronavirus replication complex formation utilizes components of cellular autophagy. J. Biol. Chem. **279**:10136-10141.

432. **Racaniello, V. R.** 2001. Picornaviridae: the viruses and their replication, p. 529-566. *In* D. Knipe and P. Howley (eds.), Fundamental virology.

433. **Ramachandran, A., J. P. Parisien, and C. M. Horvath**. 2008. STAT2 is a primary target for measles virus V protein-mediated alpha/beta interferon signaling inhibition. J. Virol. **82**:8330-8338.

434. **Ramaswamy, M., L. Shi, M. M. Monick, G. W. Hunninghake, and D. C. Look**. 2004. Specific inhibition of type I interferon signal transduction by respiratory syncytial virus. Am. J. Respir. Cell Mol. Biol. **30**:893-900.

435. **Randall, R. E. and S. Goodbourn**. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. **89**:1-47.

436. **Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz**. 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell **15**:261-267.

437. **Reeves, R. H., B. F. O'Hara, W. J. Pavan, J. D. Gearhart, and O. Haller**. 1988. Genetic mapping of the Mx influenza virus resistance gene within the region of mouse chromosome 16 that is homologous to human chromosome 21. J. Virol. **62**:4372-4375.

438. **Regad, T., A. Saib, V. Lallemand-Breitenbach, P. P. Pandolfi, T. H. De, and M. K. Chelbi-Alix**. 2001. PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator. EMBO J. **20**:3495-3505.

439. **Regad, T. and M. K. Chelbi-Alix**. 2001. Role and fate of PML nuclear bodies in response to interferon and viral infections. Oncogene **20**:7274-7286.

440. **Reichelt, M., S. Stertz, J. Krijnse-Locker, O. Haller, and G. Kochs**. 2004. Missorting of LaCrosse virus nucleocapsid protein by the interferon-induced MxA GTPase involves smooth ER membranes. Traffic. **5**:772-784.

441. **Reid, S. P., L. W. Leung, A. L. Hartman, O. Martinez, M. L. Shaw, C. Carbonnelle, V. E. Volchkov, S. T. Nichol, and C. F. Basler**. 2006. Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. J. Virol. **80**:5156-5167.

442. **Reimann, H., J. Hodges, and A. Price**. 1945. Epidemic diarrhea, nausea, and vomiting of unknown cause. JAMA **127**:1-6.

443. **Ren, J., B. Rastegari, A. Condon, and H. H. Hoos**. 2005. HotKnots: heuristic prediction of RNA secondary structures including pseudoknots. RNA. **11**:1494-1504.

444. **Reshetnyak, V. I., T. I. Karlovich, and L. U. Ilchenko**. 2008. Hepatitis G virus. World J. Gastroenterol. **14**:4725-4734.

445. **Reuter, G., H. Biro, and G. Szucs**. 2007. Enteric caliciviruses in domestic pigs in Hungary. Arch. Virol. **152**:611-614.

446. **Rezaee, S. A., C. Cunningham, A. J. Davison, and D. J. Blackbourn**. 2006. Kaposi's sarcoma-associated herpesvirus immune modulation: an overview. J. Gen. Virol. **87**:1781-1804.

447. **Rhoads, R. E.** 1993. Regulation of eukaryotic protein synthesis by initiation factors. J. Biol. Chem. **268**:3017-3020.

448. **Richards, G. P., M. A. Watson, and D. H. Kingsley**. 2004. A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. J. Virol. Methods **116**:63-70.

449. **Ricour, C., F. Borghese, F. Sorgeloos, S. V. Hato, F. J. van Kuppeveld, and T. Michiels**. 2009. Random mutagenesis defines a domain of Theiler's virus leader protein which is essential for antagonism of nucleocytoplasmic trafficking and of cytokine gene expression. J. Virol.

450. **Rima, B. K. and N. V. McFerran**. 1997. Dinucleotide and stop codon frequencies in single-stranded RNA viruses. J. Gen. Virol. **78 (Pt 11)**:2859-2870.

451. **Rivas, E. and S. R. Eddy**. 2000. Secondary structure alone is generally not statistically significant for the detection of noncoding RNAs. Bioinformatics. **16**:583-605.

452. **Rivera, V. M., J. D. Welsh, and J. V. Maizel, Jr.** 1988. Comparative sequence analysis of the 5' noncoding region of the enteroviruses and rhinoviruses. Virology **165**:42-50.

453. **Rockx, B., W. M. De, H. Vennema, J. Vinje, B. E. de, D. Y. Van, and M. Koopmans**. 2002. Natural history of human calicivirus infection: a prospective cohort study. Clin. Infect. Dis. **35**:246-253.

454. **Rodriguez, J. J., J. P. Parisien, and C. M. Horvath**. 2002. Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. J. Virol. **76**:11476-11483.

455. **Rodriguez, J. J., L. F. Wang, and C. M. Horvath**. 2003. Hendra virus V protein inhibits interferon signaling by preventing STAT1 and STAT2 nuclear accumulation. J. Virol. **77**:11842-11845.

456. **Romano, P. R., F. Zhang, S. L. Tan, M. T. Garcia-Barrio, M. G. Katze, T. E. Dever, and A. G. Hinnebusch**. 1998. Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3: role of complex formation and the E3 N-terminal domain. Mol. Cell Biol. **18**:7304-7316.

457. **Rombaut, B., R. Vrijsen, and A. Boeye**. 1990. New evidence for the precursor role of 14 S subunits in poliovirus morphogenesis. Virology **177**:411-414.

458. **Romero-Lopez, C. and A. Berzal-Herranz**. 2009. A long-range RNA-RNA interaction between the 5' and 3' ends of the HCV genome. RNA. **15**:1740-1752.

459. **Ronco, L. V., A. Y. Karpova, M. Vidal, and P. M. Howley**. 1998. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev. **12**:2061-2072.

460. **Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald**. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. J. Immunol. **175**:5260-5268.

461. **Rueckert, R. R., A. K. Dunker, and C. M. Stoltzfus**. 1969. The structure of mouse-Elberfeld virus: a model. Proc. Natl. Acad. Sci. U. S. A **62**:912-919.

462. **Ryan, M. D. and J. Drew**. 1994. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO J. **13**:928-933.

463. **Ryzhakov, G. and F. Randow**. 2007. SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. EMBO J. **26**:3180-3190.

464. **Saira, K., Y. Zhou, and C. Jones** . 2009. The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) associates with interferon regulatory factor 7 and consequently inhibits beta interferon promoter activity. J. Virol. **83**:3977-3981.

465. **Samuel, M. A., K. Whitby, B. C. Keller, A. Marri, W. Barchet, B. R. Williams, R. H. Silverman, M. Gale, Jr., and M. S. Diamond**. 2006. PKR and RNase L contribute to protection against lethal West Nile Virus infection by controlling early viral spread in the periphery and replication in neurons. J. Virol. **80**:7009-7019.

466. **Sandrock, M., M. Frese, O. Haller, and G. Kochs**. 2001. Interferon-induced rat Mx proteins confer resistance to Rift Valley fever virus and other arthropod-borne viruses. J. Interferon Cytokine Res. **21**:663-668.

467. **Sangar, D. V., S. E. Newton, D. J. Rowlands, and B. E. Clarke**. 1987. All foot and mouth disease virus serotypes initiate protein synthesis at two separate AUGs. Nucleic Acids Res. **15**:3305-3315.

468. **Sarkar, S. N., K. L. Peters, C. P. Elco, S. Sakamoto, S. Pal, and G. C. Sen**. 2004. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat. Struct. Mol. Biol. **11**:1060-1067.

469. **Sasai, M., H. Oshiumi, M. Matsumoto, N. Inoue, F. Fujita, M. Nakanishi, and T. Seya**. 2005. Cutting Edge: NF-kappaB-activating kinase-associated

protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. J. Immunol. **174**:27-30.

470. **Sasai, M., M. Shingai, K. Funami, M. Yoneyama, T. Fujita, M. Matsumoto, and T. Seya**. 2006. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. J. Immunol. **177**:8676-8683.

471. **Sato, M., N. Tanaka, N. Hata, E. Oda, and T. Taniguchi**. 1998. Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. FEBS Lett. **425**:112-116.

472. **Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira**. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J. Immunol. **171**:4304-4310.

473. **Scheuner, D., R. Patel, F. Wang, K. Lee, K. Kumar, J. Wu, A. Nilsson, M. Karin, and R. J. Kaufman**. 2006. Double-stranded RNA-dependent protein kinase phosphorylation of the alpha-subunit of eukaryotic translation initiation factor 2 mediates apoptosis. J. Biol. Chem. **281**:21458-21468.

474. **Schindler, C., K. Shuai, V. R. Prezioso, and J. E. Darnell, Jr.** 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science **257**:809-813.

475. **Schmaljohn, C.** 2007. Bunyaviridae, p. 1741-1789. *In* D. Knipe and D. Howley (eds.), Field's Virology.

476. **Schmid, D., J. Dengjel, O. Schoor, S. Stevanovic, and C. Munz**. 2006. Autophagy in innate and adaptive immunity against intracellular pathogens. J. Mol. Med. **84**:194-202.

477. **Scholthof, H. B.** 2006. The Tombusvirus-encoded P19: from irrelevance to elegance. Nat. Rev. Microbiol. **4**:405-411.

478. **Schulz, O., S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom, and Reis e Sousa**. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature **433**:887-892.

479. **Schweizer, M. and E. Peterhans**. 2001. Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. J. Virol. **75**:4692-4698.

480. **Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen**. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell **122**:669-682.

481. **Sheppard, P., W. Kindsvogel, W. Xu, K. Henderson, S. Schlutsmeyer, T. E. Whitmore, R. Kuestner, U. Garrigues, C. Birks, J. Roraback, C. Ostrander, D. Dong, J. Shin, S. Presnell, B. Fox, B. Haldeman, E. Cooper, D. Taft, T. Gilbert, F. J. Grant, M. Tackett, W. Krivan, G. McKnight, C. Clegg, D. Foster, and K. M. Klucher**. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat. Immunol. **4**:63-68.

482. **Sheth, U. and R. Parker**. 2003. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science **300**:805-808.

483. **Sheth, U. and R. Parker**. 2006. Targeting of aberrant mRNAs to cytoplasmic processing bodies. Cell **125**:1095-1109.

484. **Shisler, J. L. and X. L. Jin**. 2004. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. J. Virol. **78**:3553-3560.

485. **Silvennoinen, O., J. N. Ihle, J. Schlessinger, and D. E. Levy**. 1993. Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature **366**:583-585.

486. **Simmonds, P., A. Tuplin, and D. J. Evans**. 2004. Detection of genome-scale ordered RNA structure (GORS) in genomes of positive-stranded RNA viruses: Implications for virus evolution and host persistence. RNA. **10**:1337-1351.

487. **Simmonds, P., I. Karakasiliotis, D. Bailey, Y. Chaudhry, D. J. Evans, and I. G. Goodfellow**. 2008. Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses. Nucleic Acids Res.

488. **Skinner, M. A., V. R. Racaniello, G. Dunn, J. Cooper, P. D. Minor, and J. W. Almond**. 1989. New model for the secondary structure of the 5' non-coding RNA of poliovirus is supported by biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J. Mol. Biol. **207**:379-392.

489. **Smith, J. A., S. C. Schmechel, B. R. Williams, R. H. Silverman, and L. A. Schiff**. 2005. Involvement of the interferon-regulated antiviral proteins PKR and RNase L in reovirus-induced shutoff of cellular translation. J. Virol. **79**:2240-2250.

490. **Sobacchi, C., V. Marrella, F. Rucci, P. Vezzoni, and A. Villa**. 2006. RAG-dependent primary immunodeficiencies. Hum. Mutat. **27**:1174-1184.

491. **Sokoloski, K. J., C. J. Wilusz, and J. Wilusz**. 2006. Viruses: overturning RNA turnover. RNA. Biol. **3**:140-144.

492. **Sosnovtsev, S. V., G. Belliot, K. O. Chang, V. G. Prikhodko, L. B. Thackray, C. E. Wobus, S. M. Karst, H. W. Virgin, and K. Y. Green**. 2006. Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. J. Virol. **80**:7816-7831.

493. **Spangberg, K., L. Wiklund, and S. Schwartz**. 2000. HuR, a protein implicated in oncogene and growth factor mRNA decay, binds to the 3' ends of hepatitis C virus RNA of both polarities. Virology **274**:378-390.

494. **Spann, K. M., K. C. Tran, and P. L. Collins**. 2005. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J. Virol. **79**:5353-5362.

495. **Spitkovsky, D., S. P. Hehner, T. G. Hofmann, A. Moller, and M. L. Schmitz**. 2002. The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the Ikappa B kinase complex. J. Biol. Chem. **277**:25576-25582.

496. **Srivastava, S. P., K. U. Kumar, and R. J. Kaufman**. 1998. Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. J. Biol. Chem. **273**:2416-2423.

497. **Stack, J., I. R. Haga, M. Schroder, N. W. Bartlett, G. Maloney, P. C. Reading, K. A. Fitzgerald, G. L. Smith, and A. G. Bowie**. 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J. Exp. Med. **201**:1007-1018.

498. **Staeheli, P., R. Grob, E. Meier, J. G. Sutcliffe, and O. Haller**. 1988. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol. Cell Biol. **8**:4518-4523.

499. **Staeheli, P. and J. G. Sutcliffe** . 1988. Identification of a second interferon-regulated murine Mx gene. Mol. Cell Biol. **8**:4524-4528.

500. **Staeheli, P., F. Pitossi, and J. Pavlovic**. 1993. Mx proteins: GTPases with antiviral activity. Trends Cell Biol. **3**:268-272.

501. **Steck, F., B. Hofer, B. Schaeren, J. Nicolet, and H. Gerbe**. 1978. Equine rhinoviruses: new serotypes, p. 321-328. Equine Inf. Dis. IV.

502. **Stertz, S., J. Dittmann, J. C. Blanco, L. M. Pletneva, O. Haller, and G. Kochs**. 2007. The antiviral potential of interferon-induced cotton rat Mx proteins against orthomyxovirus (influenza), rhabdovirus, and bunyavirus. J. Interferon Cytokine Res. **27**:847-855.

503. **Stevenson, R. A., J. A. Huang, M. J. Studdert, and C. A. Hartley**. 2004. Sialic acid acts as a receptor for equine rhinitis A virus binding and infection. J. Gen. Virol. **85**:2535-2543.

504. **Strahle, L., J. B. Marq, A. Brini, S. Hausmann, D. Kolakofsky, and D. Garcin**. 2007. Activation of the beta interferon promoter by unnatural Sendai virus infection requires RIG-I and is inhibited by viral C proteins. J. Virol. **81**:12227-12237.

505. **Strauss, J. H. and E. G. Strauss** . 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58**:491-562.

506. **Stuart, A. D. and T. D. Brown**. 2006. Entry of feline calicivirus is dependent on clathrin-mediated endocytosis and acidification in endosomes. J. Virol. **80**:7500-7509.

507. **Studdert, M. J. and L. J. Gleeson**. 1977. Isolation of equine rhinovirus type 1. Aust. Vet. J. **53**:452.

508. **Studdert, M. J.** 1996. Equine rhinovirus infection, p. 211-217. *In* M. J. Studdert (ed.), Virus infections of equines.

509. **Studdert, M. J., C. A. Hartley, K. Dynon, J. R. Sandy, R. F. Slocombe, J. A. Charles, M. E. Milne, A. F. Clarke, and C. El-Hage**. 2003. Outbreak of equine herpesvirus type 1 myeloencephalitis: new insights from virus identification by PCR and the application of an EHV-1-specific antibody detection ELISA. Vet. Rec. **153**:417-423.

510. **Sugieda, M., H. Nagaoka, Y. Kakishima, T. Ohshita, S. Nakamura, and S. Nakajima**. 1998. Detection of Norwalk-like virus genes in the caecum contents of pigs. Arch. Virol. **143**:1215-1221.

511. **Suhy, D. A., T. H. Giddings, Jr., and K. Kirkegaard**. 2000. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J. Virol. **74**:8953-8965.

512. **Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell, and B. Beutler**. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc. Natl. Acad. Sci. U. S. A **101**:3516-3521.

513. **Takaoka, A., S. Hayakawa, H. Yanai, D. Stoiber, H. Negishi, H. Kikuchi, S. Sasaki, K. Imai, T. Shibue, K. Honda, and T. Taniguchi**. 2003. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature **424**:516-523.

514. **Takeuchi, O. and S. Akira**. 2007. Recognition of viruses by innate immunity. Immunol. Rev. **220**:214-224.

515. **Takeuchi, T., T. Kobayashi, S. Tamura, and H. Yokosawa**. 2006. Negative regulation of protein phosphatase 2Cbeta by ISG15 conjugation. FEBS Lett. **580**:4521-4526.

516. **Takeuchi, T. and H. Yokosawa**. 2008. Detection and analysis of protein ISGylation. Methods Mol. Biol. **446**:139-149.

517. **Taliansky, M. E. and D. J. Robinson**. 2003. Molecular biology of umbraviruses: phantom warriors. J. Gen. Virol. **84**:1951-1960.

518. **Talloczy, Z., W. Jiang, H. W. Virgin, D. A. Leib, D. Scheuner, R. J. Kaufman, E. L. Eskelinen, and B. Levine**. 2002. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. Proc. Natl. Acad. Sci. U. S. A **99**:190-195.

519. **Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre**. 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J. Virol. **74**:7989-7996.

520. **Tan, M., P. Huang, J. Meller, W. Zhong, T. Farkas, and X. Jiang**. 2003. Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. J. Virol. **77**:12562-12571.

521. **Tan, S. L. and M. G. Katze**. 1998. Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. J. Interferon Cytokine Res. **18**:757-766.

522. **Tanaka, N., M. Nakanishi, Y. Kusakabe, Y. Goto, Y. Kitade, and K. T. Nakamura**. 2004. Structural basis for recognition of 2',5'-linked oligoadenylates by human ribonuclease L. EMBO J. **23**:3929-3938.

523. **Tang, X., J. S. Gao, Y. J. Guan, K. E. McLane, Z. L. Yuan, B. Ramratnam, and Y. E. Chin**. 2007. Acetylation-dependent signal transduction for type I interferon receptor. Cell **131**:93-105.

524. **Taube, S., J. W. Perry, K. Yetming, S. P. Patel, H. Auble, L. Shu, H. F. Nawar, C. H. Lee, T. D. Connell, J. A. Shayman, and C. E. Wobus**. 2009. Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses. J. Virol. **83**:4092-4101.

525. **Taylor, D. R., S. T. Shi, P. R. Romano, G. N. Barber, and M. M. Lai**. 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science **285**:107-110.

526. **Terenzi, F., S. Pal, and G. C. Sen**. 2005. Induction and mode of action of the viral stress-inducible murine proteins, P56 and P54. Virology **340**:116-124.

527. **Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, P. J. Le, and R. L. Calderon**. 2008. Norwalk virus: how infectious is it? J. Med. Virol. **80**:1468-1476.

528. **Thackray, L. B., C. E. Wobus, K. A. Chachu, B. Liu, E. R. Alegre, K. S. Henderson, S. T. Kelley, and H. W. Virgin**. 2007. Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. J. Virol. **81**:10460-10473.

529. **Thomas, D., G. Blakqori, V. Wagner, M. Banholzer, N. Kessler, R. M. Elliott, O. Haller, and F. Weber**. 2004. Inhibition of RNA polymerase II phosphorylation by a viral interferon antagonist. J. Biol. Chem. **279**:31471-31477.

530. **Tinoco, I., Jr., P. N. Borer, B. Dengler, M. D. Levin, O. C. Uhlenbeck, D. M. Crothers, and J. Bralla**. 1973. Improved estimation of secondary structure in ribonucleic acids. Nat. New Biol. **246**:40-41.

531. **Todd, S., J. S. Towner, D. M. Brown, and B. L. Semler**. 1997. Replication-competent picornaviruses with complete genomic RNA 3' noncoding region deletions. J. Virol. **71**:8868-8874.

532. **Tough, D. F., P. Borrow, and J. Sprent**. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. Science **272**:1947-1950.

533. **Tsukiyama-Kohara, K., N. Iizuka, M. Kohara, and A. Nomoto**. 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66**:1476-1483.

534. **Tuplin, A., J. Wood, D. J. Evans, A. H. Patel, and P. Simmonds**. 2002. Thermodynamic and phylogenetic prediction of RNA secondary structures in the coding region of hepatitis C virus. RNA. **8**:824-841.

535. **Uchil, P. D. and V. Satchidanandam**. 2003. Architecture of the flaviviral replication complex. Protease, nuclease, and detergents reveal encasement within double-layered membrane compartments. J. Biol. Chem. **278**:24388-24398.

536. **Ulane, C. M. and C. M. Horvath**. 2002. Paramyxoviruses SV5 and HPIV2 assemble STAT protein ubiquitin ligase complexes from cellular components. Virology **304**:160-166.

537. **Ulane, C. M., J. J. Rodriguez, J. P. Parisien, and C. M. Horvath**. 2003. STAT3 ubiquitylation and degradation by mumps virus suppress cytokine and oncogene signaling. J. Virol. **77**:6385-6393.

538. **Umbach, J. L. and B. R. Cullen**. 2009. The role of RNAi and microRNAs in animal virus replication and antiviral immunity. Genes Dev. **23**:1151-1164.

539. **Unterstab, G., S. Ludwig, A. Anton, O. Planz, B. Dauber, D. Krappmann, G. Heins, C. Ehrhardt, and T. Wolff**. 2005. Viral targeting of the interferon-{beta}-inducing Traf family member-associated NF-{kappa}B activator (TANK)-binding kinase-1. Proc. Natl. Acad. Sci. U. S. A **102**:13640-13645.

540. **Uze, G., G. Lutfalla, and I. Gresser**. 1990. Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. Cell **60**:225-234.

541. **Valchanova, R. S., M. Picard-Maureau, M. Budt, and W. Brune**. 2006. Murine cytomegalovirus m142 and m143 are both required to block protein kinase R-mediated shutdown of protein synthesis. J. Virol. **80**:10181-10190.

542. **van der Poel, W. H., J. Vinje, H. R. van der, M. I. Herrera, A. Vivo, and M. P. Koopmans**. 2000. Norwalk-like calicivirus genes in farm animals. Emerg. Infect. Dis. **6**:36-41.

543. **van Vlijmen, H. W., S. Curry, M. Schaefer, and M. Karplus**. 1998. Titration calculations of foot-and-mouth disease virus capsids and their stabilities as a function of pH. J. Mol. Biol. **275**:295-308.

544. **Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, and G. N. Barber**. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. J. Immunol. **178**:6444-6455.

545. **Vinje, J., J. Green, D. C. Lewis, C. I. Gallimore, D. W. Brown, and M. P. Koopmans**. 2000. Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses". Arch. Virol. **145**:223-241.

546. **Vinje, J., H. Vennema, L. Maunula, C. H. von Bonsdorff, M. Hoehne, E. Schreier, A. Richards, J. Green, D. Brown, S. S. Beard, S. S. Monroe, B. E. de, L. Svensson, and M. P. Koopmans**. 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J. Clin. Microbiol. **41**:1423-1433.

547. **Vyas, J., A. Elia, and M. J. Clemens**. 2003. Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA. RNA. **9**:858-870.

548. **Wang, C., L. Deng, M. Hong, G. R. Akkaraju, J. Inoue, and Z. J. Chen**. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature **412**:346-351.

549. **Wang, Q. H., M. Souza, J. A. Funk, W. Zhang, and L. J. Saif**. 2006. Prevalence of noroviruses and sapoviruses in swine of various ages determined by reverse transcription-PCR and microwell hybridization assays. J. Clin. Microbiol. **44**:2057-2062.

550. **Wang, Q. H., V. Costantini, and L. J. Saif**. 2007. Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. Vaccine **25**:5453-5466.

551. **Wang, X., E. R. Hinson, and P. Cresswell**. 2007. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. Cell Host. Microbe **2**:96-105.

552. **Ward, J. M., C. E. Wobus, L. B. Thackray, C. R. Erexson, L. J. Faucette, G. Belliot, E. L. Barron, S. V. Sosnovtsev, and K. Y. Green**. 2006. Pathology of immunodeficient mice with naturally occurring murine norovirus infection. Toxicol. Pathol. **34**:708-715.

553. **Ward, V. K., C. J. McCormick, I. N. Clarke, O. Salim, C. E. Wobus, L. B. Thackray, H. W. Virgin, and P. R. Lambden**. 2007. Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. Proc. Natl. Acad. Sci. U. S. A **104**:11050-11055.

554. **Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan**. 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J. Virol. **80**:5059-5064.

555. **Weihua, X., S. Ramanujam, D. J. Lindner, R. D. Kudaravalli, R. Freund, and D. V. Kalvakolanu**. 1998. The polyoma virus T antigen interferes with interferon-inducible gene expression. Proc. Natl. Acad. Sci. U. S. A **95**:1085-1090.

556. **Wherry, E. J., J. N. Blattman, K. Murali-Krishna, M. R. van der, and R. Ahmed**. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J. Virol. **77**:4911-4927.

557. **Widdowson, M. A., A. Sulka, S. N. Bulens, R. S. Beard, S. S. Chaves, R. Hammond, E. D. Salehi, E. Swanson, J. Totaro, R. Woron, P. S. Mead, J. S. Bresee, S. S. Monroe, and R. I. Glass**. 2005. Norovirus and foodborne disease, United States, 1991-2000. Emerg. Infect. Dis. **11**:95-102.

558. **Wilson, J. C., J. T. Bryans, E. R. Doll, and L. Tudor**. 1965. ISOLATION OF A NEWLY IDENTIFIED EQUINE RESPIRATORY VIRUS. Cornell Vet. **55**:425-431.

559. **Wobus, C. E., S. M. Karst, L. B. Thackray, K. O. Chang, S. V. Sosnovtsev, G. Belliot, A. Krug, J. M. Mackenzie, K. Y. Green, and H. W. Virgin**. 2004. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS. Biol. **2**:e432.

560. **Wong, S. K., S. Sato, and D. W. Lazinski**. 2003. Elevated activity of the large form of ADAR1 in vivo: very efficient RNA editing occurs in the cytoplasm. RNA. **9**:586-598.

561. **Woode, G. N. and J. C. Bridger**. 1978. Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. J. Med. Microbiol. **11**:441-452.

562. **Workman, C. and A. Krogh**. 1999. No evidence that mRNAs have lower folding free energies than random sequences with the same dinucleotide distribution. Nucleic Acids Res. **27**:4816-4822.

563. **Worobey, M. and E. C. Holmes**. 1999. Evolutionary aspects of recombination in RNA viruses. J. Gen. Virol. **80 (Pt 10)**:2535-2543.

564. **Wreschner, D. H., J. W. McCauley, J. J. Skehel, and I. M. Kerr**. 1981. Interferon action--sequence specificity of the ppp(A2'p)nA-dependent ribonuclease. Nature **289**:414-417.

565. **Wullaert, A., K. Heyninck, S. Janssens, and R. Beyaert**. 2006. Ubiquitin: tool and target for intracellular NF-kappaB inhibitors. Trends Immunol. **27**:533-540.

566. **Wutz, G., H. Auer, N. Nowotny, B. Grosse, T. Skern, and E. Kuechler**. 1996. Equine rhinovirus serotypes 1 and 2: relationship to each other and to aphthoviruses and cardioviruses. J. Gen. Virol. **77 (Pt 8)**:1719-1730.

567. **Xi, J. N., D. Y. Graham, K. N. Wang, and M. K. Estes**. 1990. Norwalk virus genome cloning and characterization. Science **250**:1580-1583.

568. **Xiang, W., K. S. Harris, L. Alexander, and E. Wimmer**. 1995. Interaction between the 5'-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication. J. Virol. **69**:3658-3667.

569. **Xu, L. G., Y. Y. Wang, K. J. Han, L. Y. Li, Z. Zhai, and H. B. Shu**. 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol. Cell **19**:727-740.

570. **Yamashita, K., T. Imaizumi, K. Taima, T. Fujita, A. Ishikawa, H. Yoshida, C. Oyama, and K. Satoh**. 2005. Polyinosinic-polycytidylic acid induces the expression of GRO-alpha in BEAS-2B cells. Inflammation **29**:17-21.

571. **Yi, M. and S. M. Lemon**. 2003. 3' nontranslated RNA signals required for replication of hepatitis C virus RNA. J. Virol. **77**:3557-3568.

572. **Yokota, S., H. Saito, T. Kubota, N. Yokosawa, K. Amano, and N. Fujii**. 2003. Measles virus suppresses interferon-alpha signaling pathway: suppression of Jak1 phosphorylation and association of viral accessory proteins, C and V, with interferon-alpha receptor complex. Virology **306**:135-146.

573. **Yokota, S., N. Yokosawa, T. Okabayashi, T. Suzutani, S. Miura, K. Jimbow, and N. Fujii**. 2004. Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 contributes to inhibition of the interferon signaling pathway. J. Virol. **78** :6282-6286.

574. **Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita** . 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. **5**:730-737.

575. **Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita**. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. **175**:2851-2858.

576. **You, J., J. L. Croyle, A. Nishimura, K. Ozato, and P. M. Howley**. 2004. Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. Cell **117**:349-360.

577. **You, S., D. D. Stump, A. D. Branch, and C. M. Rice**. 2004. A cis-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. J. Virol. **78**:1352-1366.

578. **Yuan, W. and R. M. Krug**. 2001. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. EMBO J. **20**:362-371.

579. **Zahn, R. C., I. Schelp, O. Utermohlen, and L. D. von**. 2007. A-to-G hypermutation in the genome of lymphocytic choriomeningitis virus. J. Virol. **81**:457-464.

580. **Zahorsky, J.** 1929. Hyperemesis hiemis or the winter vomiting disease. Archives of Pediatrics **46**:391-395.

581. **Zhang, B., M. Li, L. Chen, K. Yang, Y. Shan, L. Zhu, S. Sun, L. Li, and C. Wang**. 2009. The TAK1-JNK cascade is required for IRF3 function in the innate immune response. Cell Res. **19**:412-428.

582. **Zhang, Y., C. W. Burke, K. D. Ryman, and W. B. Klimstra**. 2007. Identification and characterization of interferon-induced proteins that inhibit alphavirus replication. J. Virol. **81**:11246-11255.

583. **Zhang, Z. and S. Alexandersen**. 2004. Quantitative analysis of foot-and-mouth disease virus RNA loads in bovine tissues: implications for the site of viral persistence. J. Gen. Virol. **85**:2567-2575.

584. **Zhao, C., S. L. Beaudenon, M. L. Kelley, M. B. Waddell, W. Yuan, B. A. Schulman, J. M. Huibregtse, and R. M. Krug**. 2004. The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN-alpha/beta-induced ubiquitin-like protein. Proc. Natl. Acad. Sci. U. S. A **101**:7578-7582.

585. **Zhao, C., C. Denison, J. M. Huibregtse, S. Gygi, and R. M. Krug**. 2005. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. Proc. Natl. Acad. Sci. U. S. A **102**:10200-10205.

586. **Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. S. Monroe**. 2006. Norovirus classification and proposed strain nomenclature. Virology **346**:312-323.

587. **Zhou, A., B. A. Hassel, and R. H. Silverman**. 1993. Expression cloning of 2-5A-dependent RNAase: a uniquely regulated mediator of interferon action. Cell **72**:753-765.

588. **Zhou, A., J. Paranjape, T. L. Brown, H. Nie, S. Naik, B. Dong, A. Chang, B. Trapp, R. Fairchild, C. Colmenares, and R. H. Silverman**. 1997. Interferon action and apoptosis are defective in mice devoid of 2',5'-oligoadenylate-dependent RNase L. EMBO J. **16**:6355-6363.

589. **Zuker, M.** 1989. Computer prediction of RNA structure. Methods Enzymol. **180**:262-288.

590. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**:3406-3415.

591. **Zuniga, E. I., L. Y. Liou, L. Mack, M. Mendoza, and M. B. Oldstone**. 2008. Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections. Cell Host. Microbe **4**:374-386.

Appendix Original sequence data generated during project

Sequence of ERAV AHT1: position 360-7266

TGGTCGAAGGCGCTCGCAATAAGACTGGTCGTCACTTGGCTGTTCTATCATTTCAGG
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Sequence of MNV – Laboratory mouse F12: position 58-7268

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Sequence of MNV – Laboratory mouse F17: position 103-7329

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Sequence of MNV – Laboratory mouse F18: position 58-7229

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Sequence of MNV – Laboratory mouse F22: position 58-7329

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Sequence of MNV – Pet shop mouse 1: position 70-7328

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ACAGGCCGAACACTCAAACAATAATGGCTGGTGCACTTTTTGGTGCGATTGGAGGT
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Sequence of MNV – Wild rodent (*Apodemus sylvaticus*) 960: position 1-7321

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Sequence of MNV3 – Passage 0: position 42-7352

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Sequence of MNV3 – Passage 33, high moi: position 4-7351

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